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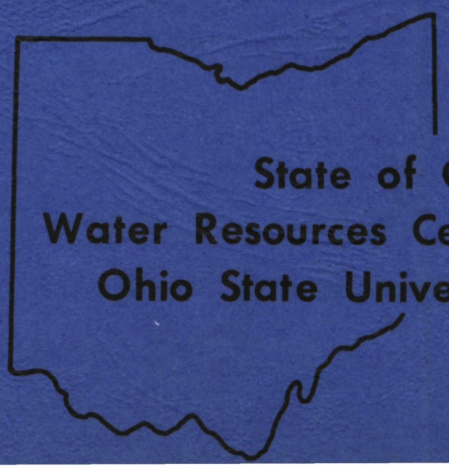
The Microbial Flora of Acid Mine Water and its Relationship to Formation and Removal of Acid

October, 1968



State of Ohio
Water Resources Center
Ohio State University

THE MICROBIAL FLORA OF ACID MINE WATER AND ITS RELATIONSHIP TO FORMATION AND REMOVAL OF ACID

Patrick R. Dugan and Chester I Randles
Assisted by
Jon H. Tuttle, Bruce McCoy and Carol MacMillan

Academic Faculty of Microbial and Cellular Biology
and the Water Resources Center of The Ohio State University

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I. INTRODUCTION

This report covers a series of continuing investigations concerning the activities of microorganisms in acidic mine water. The research was sponsored by the Office of Water Resources Research during the 27 month period from April 1, 1965 through July 1, 1967 although some of the data contained herein was acquired subsequent to this time period.

An attempt has been made to study ecological relationships between acid water and microbial activity in the field and then to verify the field observations using controlled laboratory experiments where possible.

The report is divided into five separate research chapters each of which describes a different aspect of our overall investigation. It has been compiled in this manner because each chapter represents a research paper which either has been published or will be published separately, elsewhere, in the scientific literature. A certain amount of overlap and redundancy can therefore be anticipated as the reader proceeds from chapter to chapter.

An initial cursory survey of acid polluted streams in the coal mining region of Southeastern Ohio indicated to us that similar types of microorganisms were present in all acidic streams which we sampled.

Our primary interest is in the net activity produced by the microflora and not with the taxonomic or numerical distributions of microbes per se. We therefore suspended our survey efforts and selected three field locations for further study. The three locations were:

(A) The West Branch of Racoon Creek, a stream which is consistently polluted by acid drainage (largely from gob piles) and has a pH in the range 3.5 to 7.0 depending upon environmental factors. A non-acid polluted stream (Honey Fork) merges with Raccoon Creek and therefore affords a comparative or control stream in the same geographical, geological, and other environmental circumstances. This system is further described in Chapter II.

(B) A drift mine which has a continuous flow of highly acid water (pH 2.8) that has not been exposed to field runoff, rain or other exposure to the external environment. This mine is further described in Chapter IV.

(C) An acidic stream which has been dammed and forms two lagoons, one above and one below a sawdust composition dam. This system is further described in Chapter V.

In addition to the microbial ecology considered in Chapters II, IV and V, two chapters are included that pertain primarily to ramifications and applications of the ecological studies (i.e. Chapters III and VI).

The following remarks present a brief review of some of the known reactions brought about by the iron oxidizing bacteria and the sulfate reducing bacteria in relationship to acid mine drainage. This should serve to familiarize the reader with the background upon which the research sections of this report are based.

The mineral pyrite is often found in close association with coal. When coal is mined the pyritic material is left behind as a waste product which is exposed to atmospheric moisture and oxygen. Large piles of waste pyrite

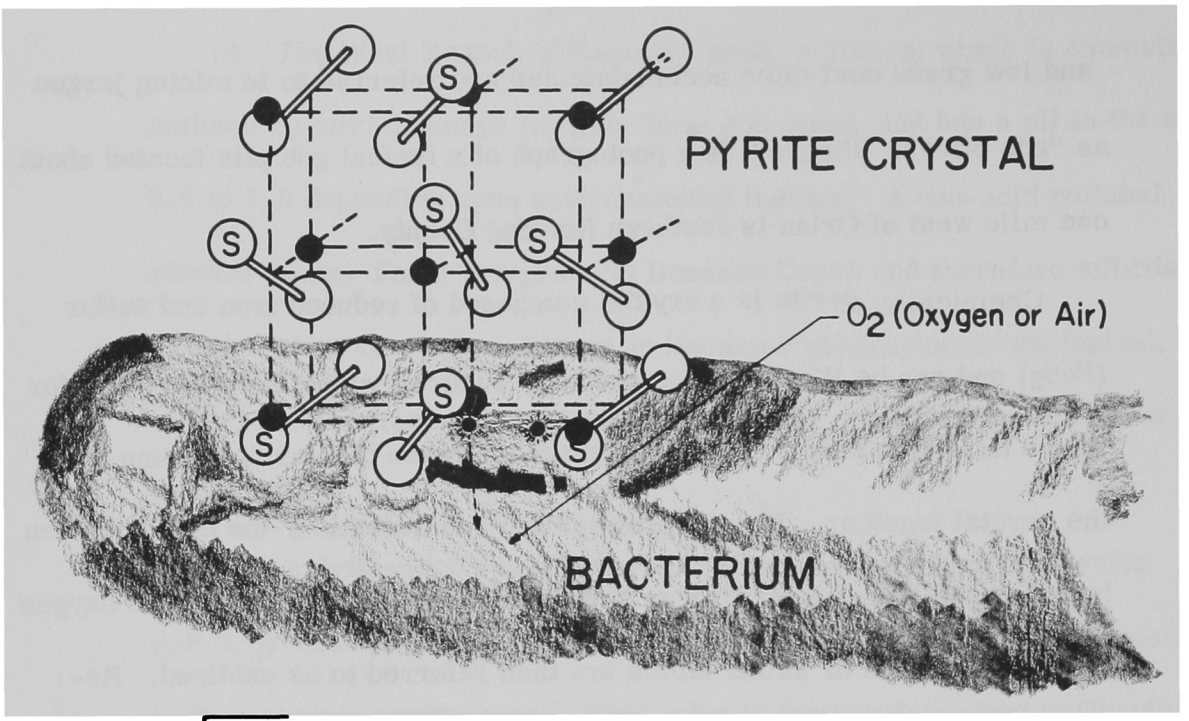
and low grade coal often accumulate and are referred to in mining jargon as "gob piles". Figure 2 is a photograph of a typical gob pile located about one mile west of Orlan is Southern Hocking County.

Chemically, pyrite is a crystal composed of reduced iron and sulfur (FeS_2) and can be illustrated as shown in Figure 1 where the ratio of sulfur atoms to iron is 2 to 1. Electrons shared between the various atoms hold the crystal together. In an oversimplified consideration, the electrons can be extracted from either iron or sulfur and accepted by atmospheric oxygen atoms. The iron or sulfur atoms are then referred to as oxidized. Removal of a single electron from the crystal, whether from iron or sulfur, destroys the integral properties of a relatively large segment of the pyrite crystal and results in an alteration of the surface area exposure.

In short hand we can indicate that ferrous (pyritic) iron will be oxidized to ferric iron by losing an electron. The ferric iron thus produced can then associate with water to form ferric hydroxide as shown in Figure 1. The hydrogen ions (H+) indicated,are acid and their presence is recognized as a lowering of the pH in the stream - a measurement of hydrogen ion concentration.

In a manner analogous to that described for iron oxidation, we can show that pyritic sulfur (Sulfide or S^{-2}) will ultimately be oxidized to sulfate (SO_4^{-2}) by losing eight electrons.

It turns out that ferric hydroxide (and there may be several other chemically complex forms of hydroxides and oxides produced) is highly colored and rather insoluble in water, so we recognize it as the colored precipitate



Iron and Sulfur Oxidizers

(Ferrous)

$$Fe^{+2} \longrightarrow Fe^{+3} + \text{(Electron)}$$

$$\underset{\text{(Ferric)}}{Fe^{+3}} + 3\,\underset{\text{(Water)}}{HO\text{-}H} \longrightarrow \underset{\text{(Hydroxide)}}{Fe(OH)_3} + \underset{\text{(Acid)}}{3H^+}$$

$$\underset{\text{(Sulfide)}}{S^{-2}\text{-}S^{-2}} + \underset{2HOH}{3O_2} \longrightarrow \underset{\text{(Sulfate)}}{2(S^{+6}O_4)} + 16 + 4H^+$$

Sulfate Reducers

$$2\,\underset{\text{(Organic)}}{\underset{OH}{R\text{-}COOH}} + SO_4 \longrightarrow 2R + CO_2 + 2OH^- + H_2S$$

Chemical

H_2S + Either Fe^{++} or Fe^{+++} → FeS (Black) + Some Oxidized S

Fig 1

Schematic representation of biological and chemical reactions involved in oxidation of pyrite crystals and subsequent reduction of sulfate.

deposited on the bottom of the acid stream, shown in Figure 3.

Now if we take another viewpoint we can consider the electrons which have been transferred as an energy transfer. It so happens that certain bacteria can extract and utilize this energy, in the form of electrons from pyrite, to partially satisfy their nutritional demands, much in the same way as people extract energy from candy. Like people, the bacteria require oxygen and water while they are carrying out the process. In other terms, pyrite is an essential nutrient for a specific type of microorganism called Thiobacillus or Ferrobacillus. The by-products formed as the result of such bacterial action on pyrite are what we recognize as acid mine pollutants. i.e. acid, ferric hydroxides and oxides, and sulfate ions. The acid and high concentrations of some ions in receiving water have a deleterious influence on other forms of life (e.g. fish) which cannot withstand or adapt to the drastic environmental change. The acid and other ions also have a highly corrosive effect on metals which renders the waters in mine areas unsuitable for most industrial and recreational purposes.

Although it is true that pyrite oxidation will take place chemically it must be pointed out that the oxidations mentioned are accelerated several hundred fold by the catalytic or enzymic action of the Thiobacillus or Ferrobacillus. bacteria. In fact, the only way these particular bacteria can grow and develop is by the utilization of reduced iron and sulfur compounds as nutrients and the very presence of these Thiobacilli or Ferrobacilli in mine waters indicates they have already been responsible for converting pyrite to acid. Relative numbers of the bacteria in waters

will therefore correlate with amounts of pyrite which must have been oxidized in order to allow that number of bacteria to grow.

Another point to be considered is that the bacteria of concern have an optimum activity in a comparitively high acid environment (pH less than 4), whereas purely chemical oxidations of reduced iron and sulfur are drastically retarded below a pH of 4.

The photograph shown in Figure 4 shows the dramatic color change that occurs during growth of these bacteria in a solution containing a reduced iron mineral in the laboratory. The blue reduced iron (ferrous) sulfate solution remained blue in the absence of bacteria while being aerated. The yellow brown solution resulted when bacteria were added to the blue solution. The color is typical of what is seen in many acid streams and results from the formation of oxidized (ferric) iron compounds.

In addition to the role of certain bacteria in the production of acid runoff, it would be worthwhile to consider the influence of microorganisms on acid mine water once it has been produced. In this regard we have been studying a system of ponds and streams in which microorganisms reduce sulfate to Sulfide and which are considered in detail in Chapter V. One stream has been impeded by a wood compost (saw dust) dam. The retarded water flow resulted in a pond upstream from the dam and the terrain was such that a lower pond was present on the downstream side of the dam.

Reduction of sulfate to sulfide is a well recognized activity of certain bacteria other than the Thiobacillus-Ferrobacillus group which led us to

investigate the pond-dam system for presence of activity of this type of organism. Preliminary data indicated that recovery of sulfate reducing microorganisms was nearly perfectly correlated with our chemical data on sulfide production.

Figure 5 is a photograph of the upper pond with the upstream side of the dam in the background. The pool is fed by an acid stream entering at the foreground. Evidence of additional acid runoff can be seen on the face of the adjacent ledge by the presence of a rusty color on the rock face.

Figure 6 shows the lower pond and gives an indication of the black color in the water which results from an iron sulfide precipitate (FeS) other than pyrite (FeS_2). This color is even more demonstrable in Figure 7, which is a close-up of the effluent from the lower pond, and serves to emphasize that the appearance of the black color is somewhat sporadic and appears to be related to climatic conditions such as temperature and stream flow rate.

We have isolated the microorganisms responsible for reduction of sulfate and are continuing to study them in our laboratory where we can control the environmental conditions. We have also isolated several microorganisms which are able to degrade the cellulose of wood and have been able to show that products of cellulose degredation (from saw dust) can serve as nutrients for the sulfate reducing bacteria. This establishes the presence of symbiotic relationships amongst the microbial flora of the dam-pond system and allows us to suggest another means of abating acid mine pollution based upon microbiologic activities. Details of this study

Figure 2. Photograph of a typical "gob" pile from which acid drainage is produced.

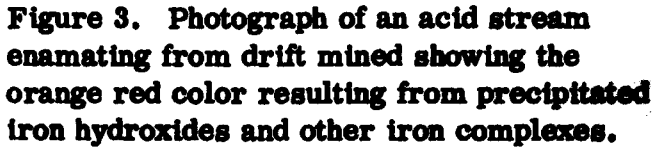


Figure 3. Photograph of an acid stream enamating from drift mined showing the orange red color resulting from precipitated iron hydroxides and other iron complexes.

Figure 4. Photograph of Cultures of Thiobacillus ferroxidans bacterium in the laboratory showing the orange red color of iron compounds produced as the result of bacterial growth. The blue container shows that the color of reduced iron ($FeSO_4$) nutrients are not altered after aeration, in the absence of bacteria, for 36 hours.

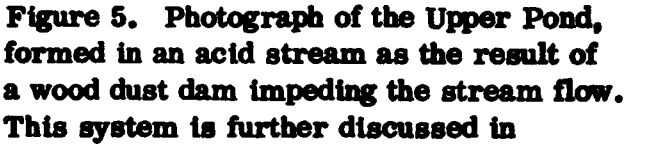


Figure 5. Photograph of the Upper Pond, formed in an acid stream as the result of a wood dust dam impeding the stream flow. This system is further discussed in Chapters V and VI.

Figure 6. Photograph of the Lower Pond formed downstream from the wood dust shown in Figure 5.

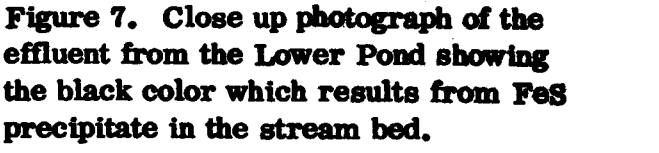


Figure 7. Close up photograph of the effluent from the Lower Pond showing the black color which results from FeS precipitate in the stream bed.

are presented in Chapters V and VI.

Reference to Figure 1 can illustrate what the sulfate reducing bacteria are accomplishing on a chemical basis. A third general group of micro-organisms, which are considered in Chapters V and VI are responsible for converting the wood dust to the organic compound illustrated. The sulfate reducers are then responsible for liberating the -COO, which is carbon dioxide gas. The H that was on the - COOH associates with the sulfur which comes from SC_4 and results in formation of hydrogen sulfide, H_2S. It is this compound which reacts with iron in the stream to form the black FeS. Notice also that we have taken an -OH off the organic molecule. This can react with acid (H^+) in the stream to form water (HOH) and results in neutralization of hhe acid. It should be realized that it takes several reactions to accomplish what has been oversimplified but this gives a fair representation of the net effects.

II. THE INFLUENCE OF ACID WATER ON AEROBIC HETEROTROPHS OF A NORMAL STREAM

High concentrations of iron, sulfate and hydrogen ions in streams located in coal mining regions are attributed to oxidation of pyritic minerals associated with coal. The oxidation of reduced iron and sulfur compounds, particularly pyrite and marcasite, has been shown to be associated with metabolic activities of certain related chemosynthetic autotrophic bacteria (3).

The autotrophic bacteria in the Thiobacillus - Ferrobacillus group are responsible for enzymic oxidation of ferrous ions and reduced sulfur compounds with concomitant production of ferric, sulfate, and hydrogen ions. Aspects of the energy metabolism of iron oxidizing autotrophs have been reported by Dugan and Lundgren (4). The role of reduced sulfur as an energy source for autotrophic bacteria has been reviewed by Peck (8). Silverman and Ehrlich have surveyed reports of microbial interactions with minerals (12).

Sulfuric acid and ferric ions thus produced have a deliterious influence on the heterotrophic biota of streams which receive the mine drainage. Ecological reports have appeared (2,6,7,10) which indicate that H_2SO_4 could cause a kill of the normal microflora of affected waters and that acidoduric species, notably fungi, appeared to thrive. Ehrlich (5) reported the isolation of large numbers of Rhodotorula from acidic copper mine effluents and described an ecological relationship between protozoa and Thiobacillus species which he cultured from the water

This is an investigation of the microbial ecology of acid mine water in

1. Published in Journal of Bacteriology 95: 1495 - 1503 (1968)

addition to a study of the influences of acid mine water on the microflora of a nonacid polluted stream. During the investigation we have considered total numbers of organisms without species differentiation based on the thesis that it is total physiological activity which contributes to the succession of events that constitute an ecosystem and that microbial activity is a primary consideration in reestablishing the biota of a "healthy" stream.

Two separate investigative approaches were used. One approach was a field survey which was conducted for the purpose of correlating bacterial types and numbers with chemical components in the stream. The second approach was to develop an artificial stream system in the laboratory The laboratory system would approximate stream conditions and allow a measure of control over variables that can not be controlled in the field. Such variables as temperature, stream flow, aeration and organic content of water all influence the number of microorganisms per unit volume of water and must be controlled before observations in the natural habitat can be interpreted.

Figure 5 is a schematic diagram of the stream system studied in the field. This system is divided into five sampling stations. Sample site B is highly acid water resulting from autotrophic bacterial activity present in a large pile of low grade coal and pyritic minerals located at position A. This mineral waste heap is often referred to as a "gob" pile. The porous mineral deposit is easily permeated with air and rain water which makes it suitable for growth of iron and sulfur oxidizing bacteria. Water flowing from B becomes somewhat diluted down stream at point C because of mixing with additional acid

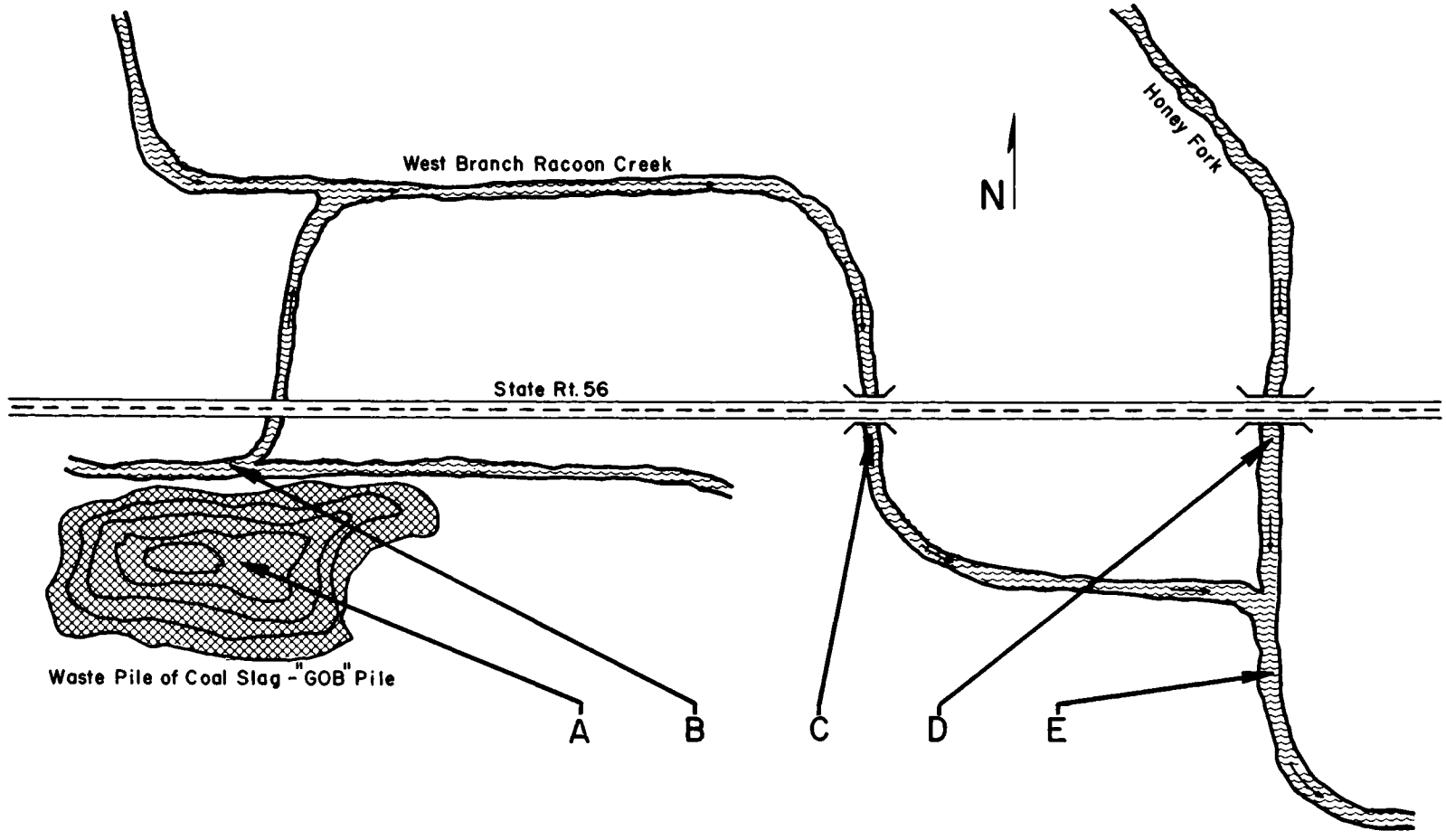


Fig 8

Schematic diagram of the stream system studied. The system is divided into five sampling stations. A represents a waste pyritic-coal slag pile. B is a highly acid stream emanating by drainage from A. C is acid water which is diluted by water same geographic area. E is referred to as a mixed stream resulting from C and D.

and nonacid drainage. An essentially neutral stream (pH 6.2 to 8.2) in the same geographic region is shown at D. Stream D flows into the diluted acid stream C and becomes stream E. E is referred to as a mixed system.

MATERIALS AND METHODS

Samples Water samples were taken in the field in sterile 8 oz bottles and held in a styrofoam cabinet until they reached the laboratory and then refrigerated at 8 C. All water samples were plated on bacteriological culture media within 24 hr after they were taken from the stream.

Chemical Determination Total dissolved iron was measured colorimetrically using the phenanthroline method according to the procedure described for a Hach Field Kit (Hach Chemical Co., Des Moines, Iowa).

Sulfate was determined turbidimetrically using $BaSO_4$ precipitate as described by the Hach procedure.

pH was determined using a Beckman pH meter

Media and Growth Conditions One ml aliquots of samples and suitable dilutions were pour plated on Tryptone Glucose Extract agar (TGE, Difco) which was supplemented with 0.5 g Yeast Extract per liter (TGYE) and on Sabouraud's Dextrose Agar (SD, Difco). The cultures were incubated at 25$\pm$ 2 C in the air for 3 days.

Anaerobic microorganisms other than Desulfovibrio were determined by adding 1.0 ml aliquots of a single tube dilution series to 9.0 ml of Thioglycollate Medium (Difco). The reciprocal of the highest dilution

showing growth after 7 days at 25 ± 2 C, as determined by appearance of turbidity in the anaerobic zone of the tubes, was taken as the number of organisms.

Sulfate reducing bacteria were enumerated using a standard three tube most probable number (MPN) method (1) according to the tube culture technique described by Postgate employing Desulfovibrio desulfuricans medium No. 3 (9). Positive tubes were black after incubation at 25 ± 2 C for 6 to 21 days.

Iron oxidizing chemoautotrophic bacteria were counted by a five tube MPN technique in the salts medium of Silverman and Lundgren (11). Positive tubes were determined after 15 days at 25 ± 2 C by presence of a dark red-brown precipitate. Uninoculated control tubes and those in which growth did not occur contained a light yellow brown precipitate at the end of the incubation period due to autooxidation of ferrous iron.

Sulfur oxidizing chemoautotrophic bacteria were enumerated in the same manner as were the iron oxidizers. One tenth g elemental sulfur per 10 ml replaced the $FeSO_4 \cdot 7HOH$ in the culture medium and positive tubes were taken as those in which sufficient acid was produced to cause 5 drops of a 1% thymol blue solution to turn red (red pH 1.2 - yellow 2.8). Control tubes and negative growth tubes gave a yellow indicator reaction.

Artificial stream system The apparatus shown in Fig. 9 was assembled to allow mixing of acid contaminated stream water and neutral stream water using controlled flow rates and mixing conditions in an attempt to simulate field conditions. Five gallon glass bottles were fitted with 3.0 mm O.D.

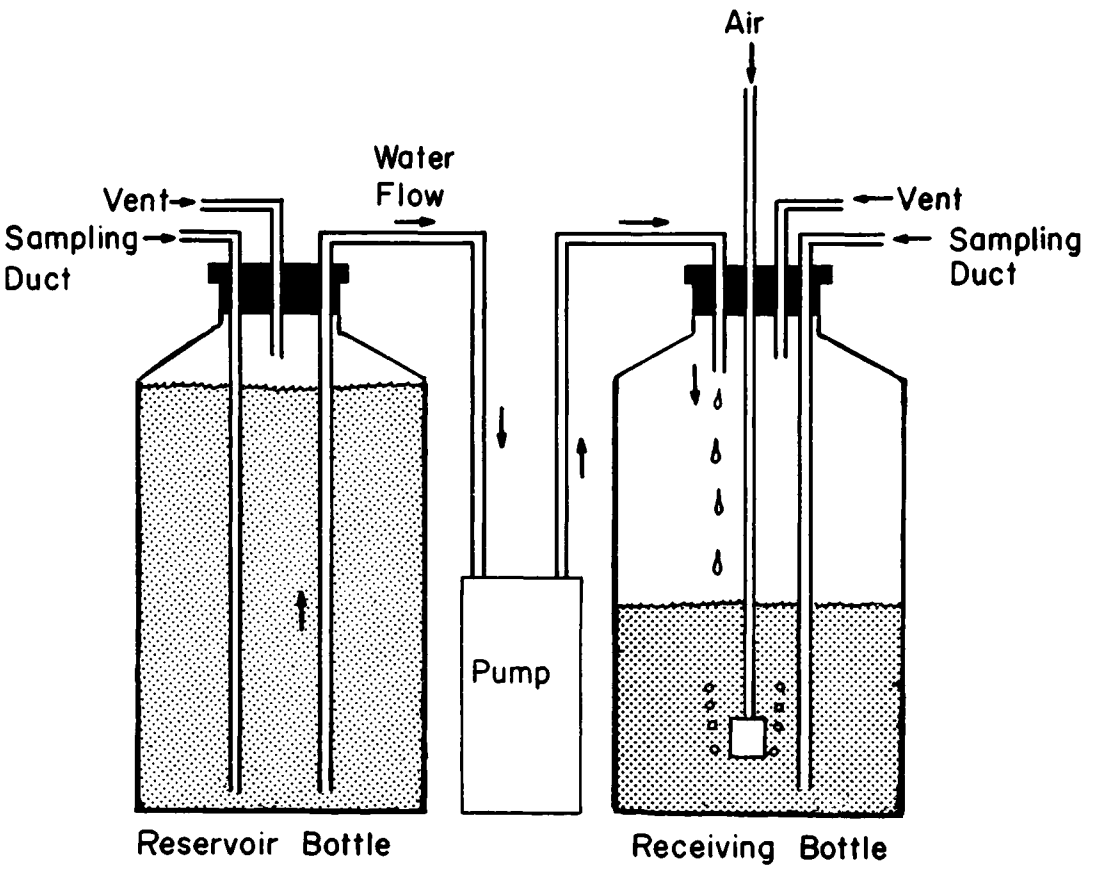


Fig 9

Apparatus used as an artificial stream system to simulate the mixing of acidic water with neutral water.

glass tubing. A peristaltic pump was calibrated to deliver a reproducible flow of water from the reservoir bottle which contained acidic mine water taken from the field at location C (Fig. 8), into the receiving bottle which was filled to 10 liter capacity with neutral stream water taken from the field at D. The receiving bottle was aerated through a glass frit dispersion tube to maintain aerobic conditions and to facilitate mixing. The pumping rate was standardized at 180 ml/hr. The receiving bottle filled after 3 days elapsed time. The system was maintained at ambient temperature (25± 2 C).

A duplicate control apparatus was used simultaneously in which neutral water from station D (Fig.) was pumped into neutral water This control accounted for changes in microbial populations in the receiving bottle which were not attributable to addition of acidic water.

Subsequent variations in water mixing procedure were used. That is, neutral water was pumped into acid water and in this case acid water was added to acid water as a control. This control accounted for changes in microbial populations in acid water which were not attributable to dilution by neutral water or pH rise.

Samples were taken at the time pumping commenced (0 time) and at random intervals over a 3 day period from all receiving bottles (i.e. 2 sample bottles plus 2 control bottles) during each experimental run.

Sulfate, iron, and hydrogen ion concentration were determined on all samples by the techniques previously described.

One ml aliquots (or appropriate dilutions) of each sample were pour

TABLE 1. Organisms, pH, sulfate, and iron concentration at the samples sites studied.

Sample SITE	pH	$SO_4^=$ CONC molar	TOTAL Fe CONC molar	S OXIDIZERS MPN/100 ml	Fe OXIDIZERS MPN/100 ml	ANAEROBES/ML THIOGLYCOLLATE	HETEROTROPHIC AEROBES/ml	
							SD AGAR	TGYE AGAR
B	**2.66** (2.5-3.0)	4.0×10^{-2} **2.3×10^{-2}** 4.8×10^{-2}	4.1×10^{-3} **7.5×10^{-4}** 9.0×10^{-3}	**1.75×10^{5}** (2.10×10^{2}-7.9×10^{5})	**6.89×10^{4}** (2.8×10^{3}-3.5×10^{5})	0	**15.1** (1-49)	**19** (1-65)
C	**3.23** (2.5-4.3)	4.5×10^{-3} **2.6×10^{-3}** 6.7×10^{-3}	2.1×10^{-4} **7.2×10^{-5}** 5.2×10^{-4}	**2.20×10^{4}** (2.1×10^{2}-1.3×10^{5})	**3.60×10^{2}** ($0.1.3\times10^{3}$)	**4.4** (1-10)	**105** (8-300)	**87** (28-150)
D	**6.60** (6.2-8.2)	4.3×10^{-4} **2.2×10^{-4}** 5.2×10^{-4}	1.4×10^{-5} **7.2×10^{-6}** 1.8×10^{-4}	**5.80×10^{2}** (0-3.3×10^{3})	**1.01×10^{2}** (0-4.26×10^{2})	**2800** (100-10000)	**5,776** (86-17,000)	**3,379** (762-7000)
E	**3.23*** (2.6-4.9)	2.1×10^{-3} **1.5×10^{-3}** 2.8×10^{-3}	1.0×10^{-4} **2.1×10^{-5}** 1.8×10^{-4}	**7.32×10^{3}** (4.9×10^{2}-1.3×10^{4})	**8.03×10^{4}**** (8.0×10^{1}-3.2×10^{5})	**10** (10-10)	**1,371** (63-3,900)	**1,257** (220-3000)

Bold numerals give average values.
Numbers in Brackets indicate ranges.

*This average value is probably biased because of a single low result at the lower range limit.
**This average value is probably biased because of a single high result at the upper range limit.

plated on SD agar and TGYE agar. All plates were incubated at 28 ± 2 C in air for 3 days and colonies were enumerated.

Fresh water was brought to the laboratory from the field for each experiment. Therefore, control values differ in each experiment (See Fig. 10 Fig. 12 and Table 1), because of changing conditions in the field.

RESULTS

An initial survey of acidic streams over a several square mile area indicated that bacterial and yeast populations at the various sites were similar with respect to types of colonies on the plating media used.

The values presented in Table 1 represent a compilation of data taken at the locations shown in Fig. 8 . The data represent an average of a minimum of 6 values. Comparison of numbers of heterotrophic microorganisms counted on either TGYE or SD shows that fewer microbes were present in water as acidity increased from locations D to C to B.

Figure 3 presents data obtained from the artificial stream system and shows that the actual pH of the mixture, when acid water (C) was added to neutral water (D), did not decrease as rapidly as predicted by simple dilution (theoretical curve). The theoretical curves plotted in Figs. 10 and 12 show anticipated ion concentrations in the receiving bottles. All theoretical curves were calculated from equation 1 (page 26).

Buffering capacity of neutral water in the mixed stream system is demonstrated in Fig. 11. A 100 ml sample of water obtained at site D (Fig. 8)

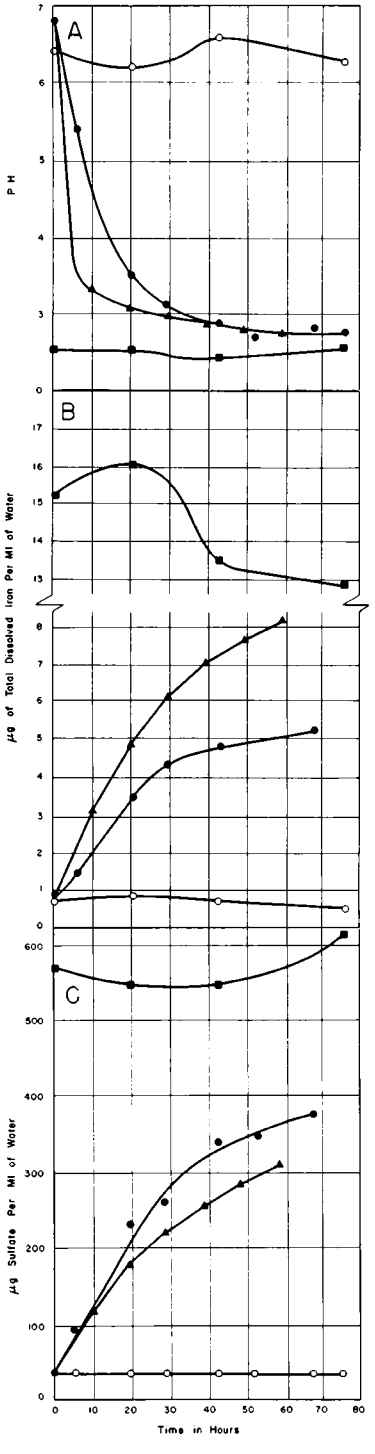


Fig 10

Changes in (A) pH, (B) dissolved iron and (C) sulfate in an artificial stream system when acidic water was added to neutral water. ○ neutral water control ● mixture resulting from addition of acid water to neutral water. ▲ calculated theoretical curve. ■ acid water control.

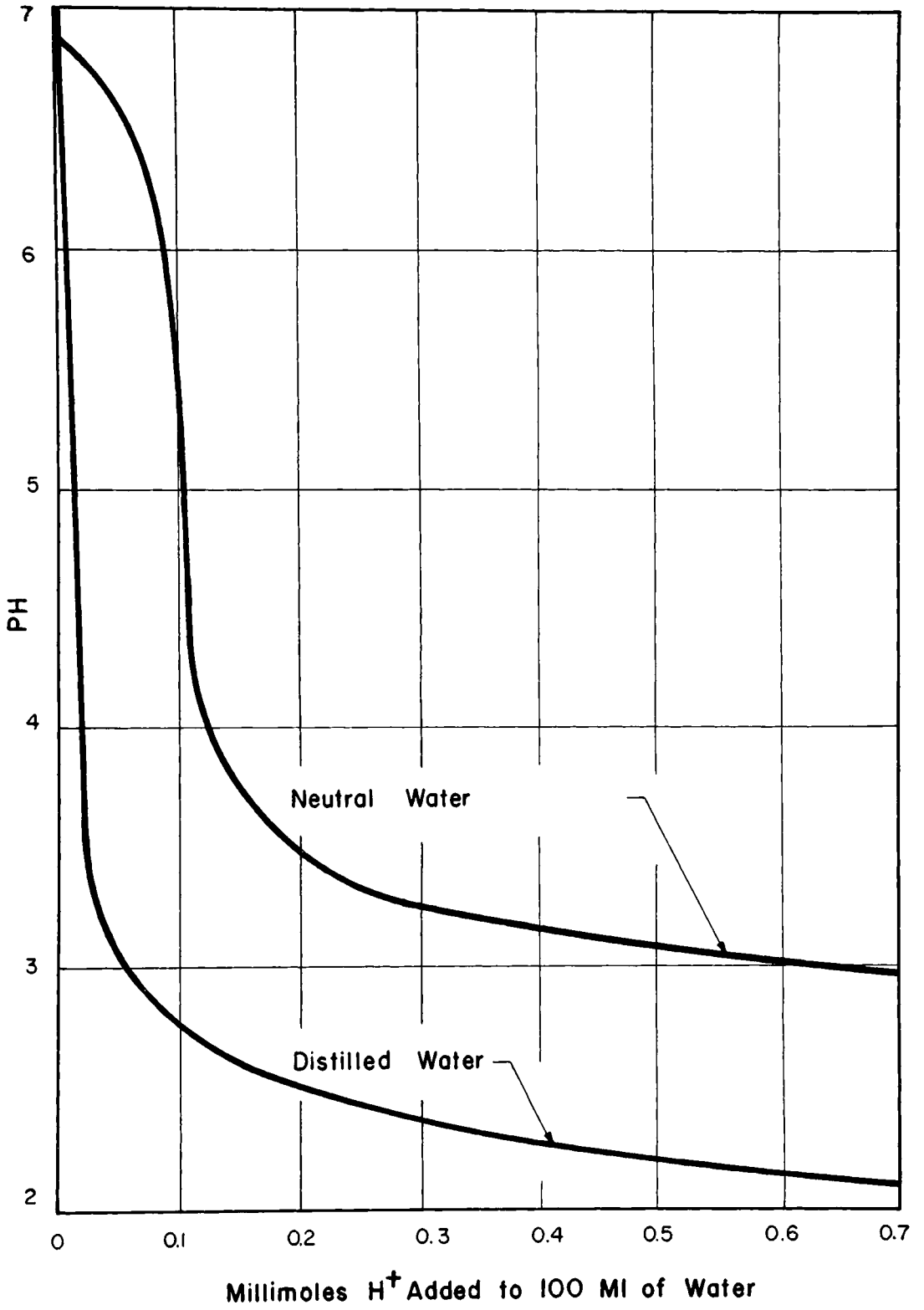


Fig 11

Curves showing change of pH in distilled water and neutral stream water when titrated with 0.02 N_2H_4 SO .

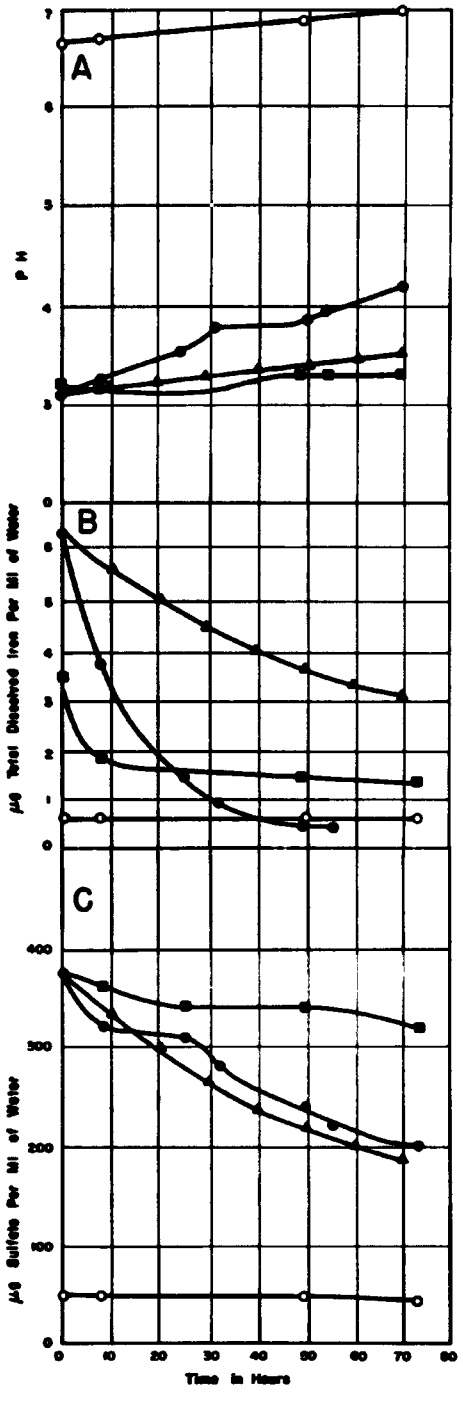


Fig 12

Changes in (A) pH, dissolved iron and (C) sulfate in an artificial stream system when neutral water was added to acidic water. O neutral water control, ● mixture resulting from addition of neutral water to acid water.

▲ Calculated theoretical curve.

■ Acid water control.

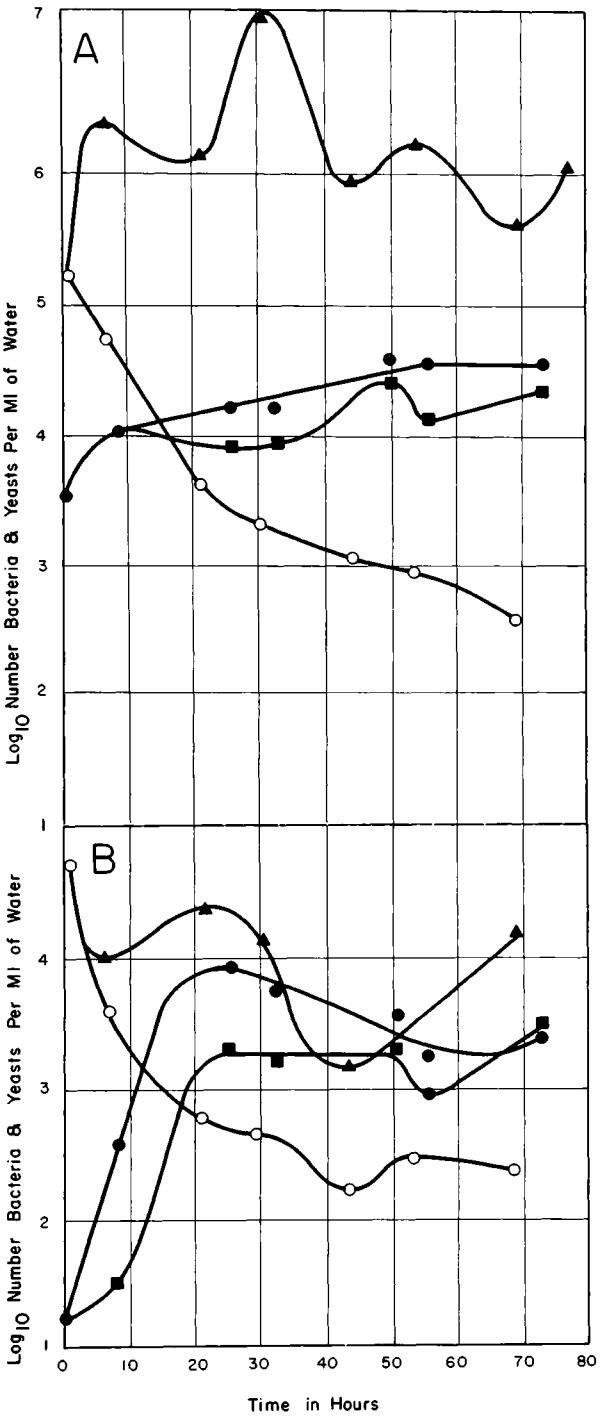


Fig 13

Enumeration of heterotrophic aerobic microorganisms in an artificial stream system as determined on (A) tryptone glucose yeast extract agar and (B) Sabouraud's agar. O in mixture resulting from acid water added to neutral water. ● in mixture resulting from neutral water added to acid water. ▲ neutral water control. ■ acid water control.

Equation 1

$$\text{Theoretical ion concentration in receiving bottle} = \frac{\text{average concentration in reservoir bottle} \times \text{Liters of water pumped during from T-10 hr to T hr 10 hr}}{\text{Total liters of water in receiving bottle at T hr}} + \frac{\text{Theoretical concentration in the receiving bottle calculated at previous time interval (T-10 hr)} \times \text{Liters of water in receiving at T-10 hr}}{\text{Total liters of water in receiving bottle at T hr}}$$

was titrated with 0.02 N H_2SO_4.

Figure 12 illustrates the changes in pH, dissolved iron and sulfate in mixed water when neutral water was added to 10 liters acid water at the rate of 180 ml/h4 for a period of 3 days.

Figure 13 shows the survival of heterotrophic aerobic microorganisms recovered on TGYE and SD agar while either neutral water was being added to 10 liters of acid water at the rate of 180 ml/hr or acid water was being added to neutral water at the same rate.

DISCUSSION

Data presented in Table 1 suggest that the heterotrophic microflora which can be recovered from an acid stream on an acid growth medium (SD agar) probably represents transient organisms which are not indigenous to the acid stream. For example, an average 5776/ml was found in a relatively nonacid stream. When this water was mixed with acid water in approximately equal volumes, the average recoverable heterotrophic population dropped by 78%. The dilution can be approximated by the change in sulfate and iron concentration when stream C mixes with stream D to form B. No significance is attached to differences between values obtained on TGYE agar as compared to SD agar. The low number of organisms recovered from locations B and C suggests that a small population of transient heterotrophs is tolerant to the acid environment but do not proliferate there.

Anaerobic bacteria were present in neutral waters, in small numbers (10 or fewer/ml) in diluted acid water (C, E) and absent in highly acid water

(B), which suggests they were not acid tolerant. This may be due to: i. the high Eh of acidic waters which results from high concentrations of oxidized compounds, ii. a low concentration of organic material, iii. the absence of those microorganisms that utilize oxygen while metabolizing organic materials. On two occasions, the anaerobic species isolated from diluted acid water included sulfate-reducing bacteria. Sulfate-reducing organisms were either lacking or present in very small numbers in acid water. This probably precludes biological sulfate removal from these streams under natural conditions; no evidence of the sulfate removal process was observed.

The predominating aerobic heterotrophic microorganisms in the acid water were yeasts and molds with a different gram-negative bacilli recovered. The bacilli were generally non-motile, oxidase negative, aerobic or facultative species. The bacteria formed white or cream colored colonies on the media employed. One aerobic oxidase positive bacterium was also isolated. The organisms have not been thoroughly characterized but are tentatively identified as Achromobacter and Pseudomonas. No gram-positive bacteria were over isolated from the acid streams using the techniques employed. The organisms examined appear to be transients entering from a nonacid environment. We must therefore presume that the gram-negative bacteria have a greater permeability barrier to hydrogen ions in the environment which gives the gram-negatives survival advantage. The permeability barrier may be related to the higher lipid content of the gram-negative cell envelope as compared to that of the cell wall of gram positive bacteria.

Comparison of the iron and sulfur oxidizing chemosynthetic autotrophic bacteria indicates that the sulfur oxidizers were nearly always present in greater numbers by a factor of 10 than were the iron-oxidizing bacteria. Since the iron oxidizers also oxidize sulfur, the counts of sulfur-oxidizing bacteria may represent both types. The difference can also be attributed to the increased energy available in reduced sulfur compounds over that of ferrous iron. The concentration of sulfate was always greater than 10 times that of iron in the same water. The difference between sulfate and iron concentrations has usually been attributed to removal of iron by precipitation as ferric hydroxides. However, the observed high sulfate concentration may support the views expressed by Silverman and Ehrlich (12), in that oxidation of Fe^{++} will result in formation of $SO_4^{=}$ from $S^{=}$.

Numbers of iron and sulfur oxidizers appeared to have a positive correlation with acid concentration. Presence of these bacteria in the neutral stream probably indicates contamination by acid effluents from an unobserved source. The data also suggest that the autotrophic bacteria are tolerant to average pH values up to 6.6. Sulfur oxidizers in general have a wide pH range.

Values of the various parameters measured in the field had a wide variance which can be attributed to daily fluctuations in uncontrolled environmental variables. For example, rainfall can cause a sudden increase in cell numbers in a stream as a result of runoff from surrounding land. The increase in cell numbers would be followed by a sudden decrease in numbers

because of dilution. This is particularly true of the autotrophic bacteria which appear to proliferate in the "gob" pile (Fig. 8, A). The organisms as well as their metabolic by-products; acid, sulfate, and iron ions, are flushed out of their waste pile during a heavy rain, causing a sudden surge in concentration in the stream. If the rainfall is prolonged the reservoir in the waste pile is depleted and the concentrations of acid, sulfate and iron ions are markedly diluted in the stream.

A laboratory investigation was conducted for the purpose of controlling environmental variables to more accurately assess the field observation.

Figure 10shows that the actual iron concentration in the receiving bottle did not increase as rapidly as predicted. Sulfate concentration increased more rapidly than expected, to a maximum of about 65 µg/ml greater than theoretical. An explanation may be: i. neutral water acts as a buffer in the mixed water system, ii. iron precipitates from solution, and iii. sulfate is formed in the mixed water probably as the result of oxidation of reduced sulfur compounds contributed by the neutral water. Figure 11demonstrates a buffering effect by neutral water over the entire pH range of the titration. It must be emphasized, however, that buffering capacity probably varies with environmental conditions in the field. The second possibility is not unexpected, since iron was observed to precipitate as yellow-orange material on the bottom of the receiving bottle. As indicated in Fig. 10 B iron also precipitated in the acid water control (reservoir). This factor influences the actual iron concentration in the mixture since, as time progressed, less

iron/hr was added to the receiving bottle. The calculations involved in the determination of the theoretical curve (see equation 1) allow for this factor. The difference between the actual and theoretical iron concentrations can then be attributed to precipitation of iron in the receiving bottle. The unexpected increase in the sulfate concentration in the mixture (Fig.10C) is attributable to changes in the mixture and not in the acid water contained in the reservoir bottle. The sulfate concentration in the neutral water control remained constant throughout the experiment. The discrepancy between actual and calculated curves may be due to biological as well as chemical and physical factors. Production of sulfate via microbial oxidation of reduced sulfur compounds in the neutral water is a possibility. Sulfate may also be released from precipitated ferric iron resulting from variation in pH.

When neutral water (D) was added to acid water (C), (Fig.12) the actual pH in the mixture increased more rapidly than the theoretical pH. This is consistent with the observed buffering capacity of the neutral water. The flat portion of the actual pH curve between 32 and 45 hr probably indicates oxidative production of hydrogen ion which compensates for an anticipated rise in pH due to dilution by neutral water.

The iron concentration (Fig.12B) in the mixed water (receiving bottle) decreased more rapidly than predicted by dilution of the acid water with neutral water. Virtually all of the iron in the mixture precipitated as the pH increased. This is probably due to oxidation of ferrous to ferric iron and the decreased solubility of ferric iron with increasing pH. The iron concentration

of the neutral water control remained constant throughout the experiment. Precipitation occurred rapidly during the first 8 hr in the acid water control, and at a slower rate after 8 hr.

After 15 hr, the actual sulfate concentration in the mixture (Fig.12C) was greater than could be accounted for on the basis of dilution. No additional sulfate was produced in the acid water alone and the data obtained in this mixed system is in accordance with the data obtained when acid water was added to neutral water, (Fig. 3C).

In the artificial stream system, numbers of microorganisms in the neutral water controls fluctuated with time although they were generally present at 10^6 per ml or greater. Comparison of the neutral control curve shown in Fig.13A (approx. 10^6/ml) to that in Fig. 6B (approx. 10^4/ml) suggests that less than 10% of the organisms in neutral water are tolerant to the acidity of SD agar (pH 5.5). This observation is verified by comparing the sharp drop in numbers of organisms over the first 20 hours when acid was added to neutral water to the change in pH shown in Fig.10A. A plot of the total counts of organisms vs time appears quite similar to the pH vs time plot.

Numbers of organisms/ml of acid water control increased during the first 20 hr (Fig.13B) which shows that the acid water (pH 2.5) was able to support growth of organisms up to a level of about 2×10^3/ml. It is interesting to note that addition of neutral water to acid water results in organism counts stabilizing at about 5×10^4/ml (Fig.13A) whereas the counts were

approximately 10^3 when acid water was added to neutral water This again indicates that the acid water contains an acid tolerant microflora which is maintained when neutral water is added and relatively few acid tolerant microbes enter the system with the neutral water Acid water added to neutral water results in a lower stabilization value which is related to the relative number of acid tolerant microbes present.

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III. THE RELATIVE INFLUENCE OF IRON, SULFATE AND HYDROGEN IONS ON THE MICROFLORA OF A NON-ACID STREAM[1]

We are all here in recognition of the fact that coal mine drainage is a serious water problem. Our business is to consider means of either preventing the problem, or in cases where the problem is not readily amendable to prevention (eg. abandoned drift mines), we must look for methods of abatement. In the latter situation we must determine how to treat the acid water under a variety of circumstances and we must also determine the extent of abatement that is necessary.

Many of us are convinced, on the basis of sound experimental data, that a large proportion of the acidity and other ions in mine drainage is there as the result of metabolic activities of autotrophic bacteria acting on pyritic minerals. This conclusion is well documented elsewhere (1,2,3). This being the case, one can predict that much of the problem could be prevented by inhibiting the bacterial activity if we kenw how to get at the bacteria in the mines. With reference to abatement we also have presented data (4,5) which shows that a significant measure of iron, sulfate, and hydrogen ions can be removed from mine water via metabolic activities of other groups of microorganisms -- particularly Desulfofibrio and Desulfotomaculum species.

The influence of acidic mine drainage on the microflora of "normal" streams is the present consideration; and this is related to the extent of abatement required to recover acid polluted streams.

1. Published in: Proceedings of 2nd Coal Mine Drainage Research; Mellon Inst. Pittsburgh, Pa., pp. 64-79 (1968).

We have been conducting a continuing investigation of the metabolic activities and ecological relationships among microorganisms in both acidic mine effluents and in streams exposed to mine effluents.

"The Influence of Acid Water on Aerobic Heterotrophs of Normal Streams" has been reported elsewhere (6). A brief review of previous data should help to introduce the approach to the problem we wish to present in this paper. We were able to show that acid streams contained relatively low numbers of acid tolerant aerobic heterotrophic microbes which appeared to originate in non-acid effluents such as field runoff. The acid tolerant heterotrophs increased in numbers to a maximum of about 2×10^3/ml in acidic streams, until the pH decreased to approximately 3.0. These organisms then represented the heterotrophic aerobic microflora of streams which were comprised of a mixture of acid drainage and non-acid water. A stream which was composed entirely of acid drainage did not have a similar microflora. Most Gram (+) aerobic and anaerobic bacteria died out very rapidly in acidic water and comprised a very small percentage of the total microbial population. Iron and sulfur oxidizing autotrophic bacteria were always present in mine water and in streams contaminated by mine water.

Our present objective is an attempt to assess the relative influence of three of the prominent chemical parameters that constitute acid mine drainage, as they effect the aerobic heterotrophic microflora of receiving streams. The approach is based on the premise that physiological activity of microorganisms is fundamental in triggering the ecological succession which ultimately results

in re-establishment of higher forms of life (eg. insects, fish, etc.) in streams. Although iron, sulfate and hydrogen ions are all characteristic of acidic mine effluents, they appear to vary independently with respect to concentration in drainage which originates at different locations. It therefore becomes important to determine (A) which component has the most deliterious influence on the biota, (B) at what concentration the ion is deliterious and (C) whether or not significant interactions exist among ions as they influence biological processes. These considerations have practical significance in that they could help to determine levels of abatement that are essential to recovery of "polluted" streams by natural processes. We also wish to know if a recognizable point exists at which growth of a normal aquatic microflora is inhibited and/or killed. This would allow prediction of the relative amount of acid pollution a stream could tolerate in a variety of circumstances and also to what extent we must treat the water.

A systems approach was used in our experimental procedure.

Heterotrophic aerobic bacteria were isolated from a non-acid contaminated stream (pH 6.2 to 8.2) which is located in the immediate vicinity of several acid contaminated streams in Southeast Ohio (pH 2.4 to 4.9). Total numbers of aerobic heterotrophic microorganisms present in stream water were also enumerated by standard dilution and plate counting techniques using Tryptone Glucose Yeast Extract Agar (Difco). Three isolates were selected for study and were identified as: Pseudomonas isolate M-1, Flavobacterium isolate M-2, and Bacillus isolate M-3. We also studied Pseudomonas isolate O.S.U. 229 which had been isolated and

described as an acid tolerant bacterium prior to this investigation.

A growth medium was developed which allowed each of the four isolates to be maintained at growth levels equivalent to total counts of microorganisms found in the field (non-acid water) when cultivated in the apparatus shown in Figure 14 The culture vessel was a 20 liter glass carboy which contained 10 liters of culture medium. Figure 15 represents a comparison of Pseudomonas M-1 growth in two different defined growth media and sterilized stream water (HF) as determined by plate counts on Tryptone Glucose Yeast Extreact Agar. The growth curve for synthetic medium (S1) nearly parallels growth in natural Honey Fork Creek water (HF). Similar experiments using the other organisms selected for study gave similar results. On the basis of data obtained from several experiments of this type; the culture medium listed in Table 2 was selected for all further studies.

Sulfate, iron, and hydrogen ions were assumed to be the chemical parameters which are most characteristic of acidic mine drainage. The effect of each of these chemical variables on growth and inhibition of each of the four bacterial isolates was examined after 72 hours incubation at 22°C ± 2 in the experimental system described.

pH was varied by addition of either HNO_3 or NaOH to give final values in unit increments from pH 2 to pH 7 inclusive.

Iron ion content was adjusted by addition of ferric nitrate ($Fe(NO_3)_3$ 9 HOH) to give final ferric ion concentrations of 1, 25, 100, and 500

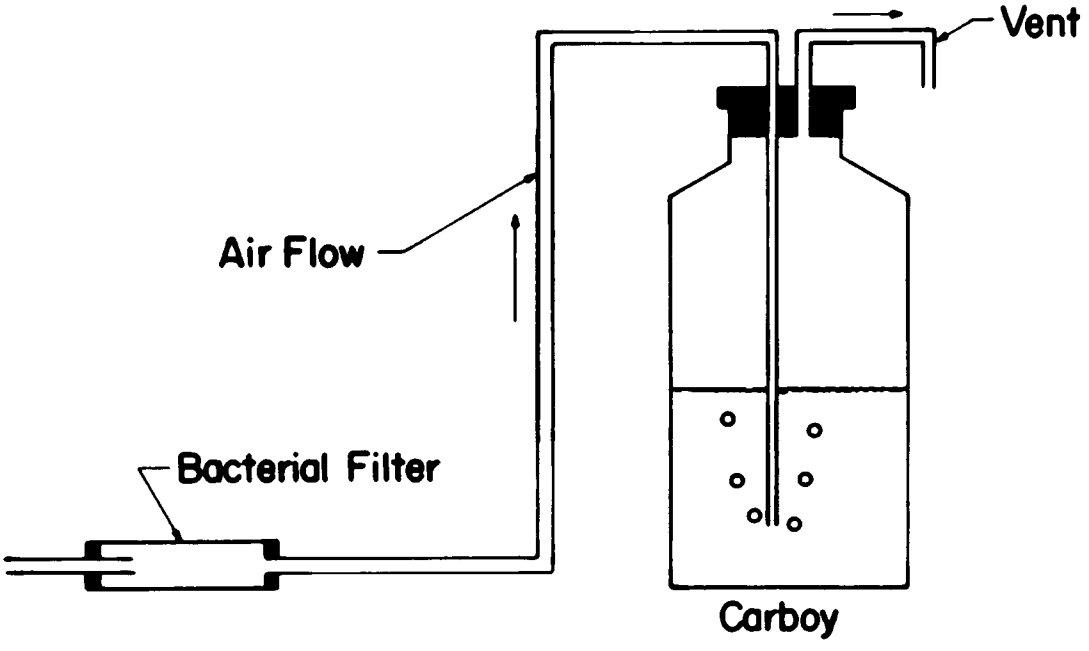

Fig 14

Diagram of apparatus used to cultivate organisms in stream water and to develope suitable growth medium.

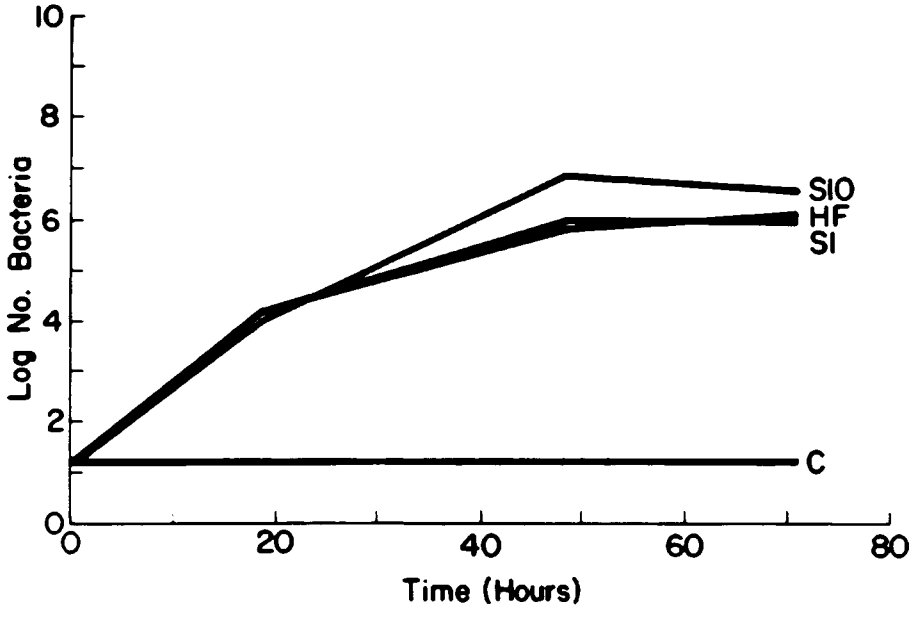

Fig 15

Comparison of growth of Pseudomonas M-1 in Honey Fork stream water (HF), defined growth medium (S1), and a tenfold increase in organic content of the defined growth medium (S10).

TABLE 2

COMPONENTS OF THE DEFINED CULTURE MEDIUM

Element	Concentration in ug/ml	Added as
A^{+++}	1	$Al_2O_3 \cdot 2H_2O$
Ca^{++}	10	$CaCl_2$, $Ca(NO_3)_2$ $4H_{20}$, $CaSO_4 \cdot 2H_2O$
Fe^{+++}	1	$FeCl_3$ $6H_2O$
Mg^{++}	16.4	$MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 6H_2O$
Mn^{++}	0.2	$MnCl_2 \cdot 4H_2O$
Na^{+}	30	$NaHCO_3$, Na_2HPO_4, Na_2SO_4
K^{-}	20	$K_2CO_3 \cdot 2H_2O$ KF, K_2SO_4
HCO_3^{-}	75	$NaHCO_3$
CO_3^{--}	11.5	K_2CO_3 $2H_2O$
Cl^{-}	56.6	$MnCl_2 \cdot 4H_2O$ $MgCl_2$ $6H_2O$ $FeCl_3 \cdot 6H_2O$, $CaCl_2$
F^{-}	0.8	KF
NO_3^{-}	10	$Ca(NO_3)_2$ $4H_2O$
PO_4^{---}	2	Na_2HPO_4
SO_4^{--}	16	Na_2SO_4, K_2SO_4, $CaSO_4$, $2H_2O$
SiO_2	15	$2SlO_2$ $2H_2O$
Trypticase Soy Extract	1	(Difco medium)

micrograms per ml of medium.

Sulfate ion content was adjusted by addition of sodium sulfate (Na_2SO_4) to give final sulfate concentrations of 50, 500, 1000, and 5000 micrograms sulfate ion per ml of medium.

In order to experimentally examine the influence of three variables at several concentrations on growth of bacteria in a simulated environment, a single all inclusive experiment was deemed necessary. This was considered particularly important because statistical significance and measures of interaction were to be calculated and minor experimental variations could not be tolerated. Ninety six separate cultivation flasks were required for the experiment, which precluded the use of 20 liter carboys. For this reason, 250 ml Erlenmeyer flasks, each containing 100 ml medium, were assembled as shown in Figure 16.

Table 3 summarizes the experimental procedures described, which were used to study the effect of each chemical variable (i.e. $SO_4^=$, Fe^{+3}, H^+) on growth and inhibition of each of the four bacterial isolates.

DATA ANALYSIS

These data representing survival of each microogranism cultivated in the described experimental system were analyzed statistically using the O.S.U. MR 90 program which is a complete package containing its own input and output routines and is included in the O.S.U. System Subroutine Library tape for the IBM 7094 computer and operating system. The program

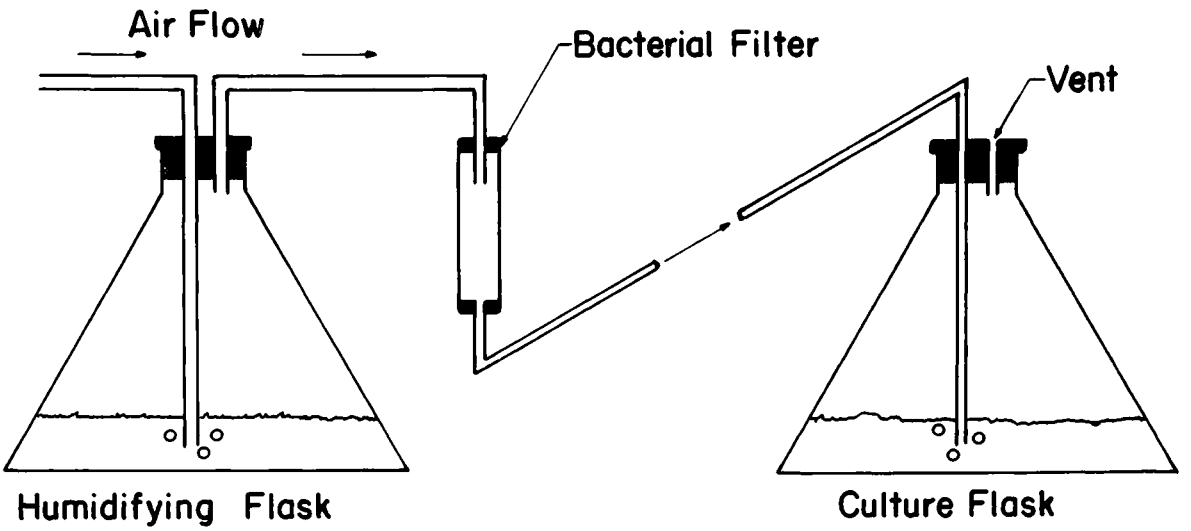

Fig 16

Diagram of apparatus used to cultivate organisms in final experiment for the purpose of statistical analysis.

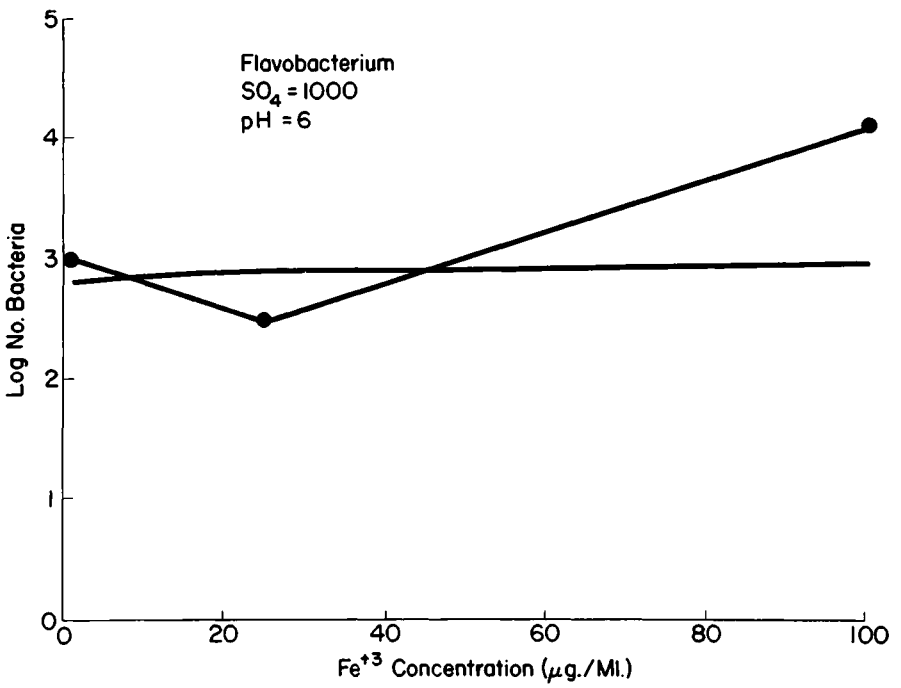

Fig 17

Plot of cell numbers vs dissolved iron concentration under the conditions specified.

TABLE 3

Summary of Experimental Procedure Employed in the Study

1. Non Acid Stream Water
   - → Total counts of aerobic heterotrophs
   - → Select 3 representative species
     + Pseudomonas 229
2. Develop a defined growth medium which simulated the stream nutritionally and which would allow the four bacterial species to be maintained at growth levels equivalent to total counts found in the field when cultivated under forced aeration.
3. Systematically vary the simulated stream water by addition of $SO_4^{=}$, Fe^{+++}, and H^{+}, and follow growth of individual organisms.
4. Statistically correlate survival of bacteria as dependent variable and ion concentrations as independent variable.
5. Develop mathematical models and generate curves which fit experimental data.
6. From the models, test hypothesis involving relationships and interactions among parameters for each microorganism.

was called and driven by the WRTR driving deck which operates through the O.S.U. 7094 operating system only. The data analysed encompassed the following independent variables: sulfate ion, 50-5000 µg/ml.; ferric ion, 1-100µg/ml.; and pH 2-7. Significant amounts of iron precipitated from solution at pH levels above three if concentrations greater then 100 µg/ml. were originally introduced. These values were not included in the statistical analysis. Survival numbers of microorganisms under the experimental conditions were then used as the dependent variable.

Functions of the independent variables were sought which would generate curves closely approximating the values of the dependent variables. To assist in discovering significant relations among the parameters a correlation analysis was performed between the log number of organisms and other variables which might be of interest (e.g. pH, $(pH)^2$, log (Fe^{+++}), log (SO_4^{--}), and pH/log (SO_4^{--})). Results close to plus or minus one indicates that a correlation exists. A value close to zero indicates a lack of correlation.

The results of the correlation analysis indicate that significant correlations exist between the log number of organisms and the variables pH, $(pH)^2$, and $(pH)^3$ That is, the values fall in the range of 0.8 to 0.9 on a correlation scale of minus one to plus one.

The log numbers of organisms from replicate plates were correlated with each other for the Flavobacterium (M2), Pseudomonas 229, and the Pseudomonas (M1). The values were 0.986, 0.994, and 0.995 respectively. Bacillus was not included because we could not derive sufficient data from the test system for

programming.

On the basis of correlation studies and hypothesized interrelationships a series of mathematical models was developed. The models were developed to generate curves which fit the experimental data. That is, a formula (model) was developed which gives the best fit to the experimental data for each organism investigated.

A series of hypotheses, involving relationships of the parameters, was made and tested. On the basis of statistical significance using F and t tests, the hypotheses was retained or deleted from the model.

Figures 17, 18 and 6 present some of the data showing relationships observed when Flavobacterium M2 was the test organism. Figure 4 shows a plot of cell numbers versus iron concentration when the pH was 6 and the sulfate concentration was 1000 µg/ml.

Figure 18 shows cell numbers vs sulfate concentration at pH 6 and 25 µg Fe/ml of medium.

Figure 19 shows the influence of pH on cell numbers when Fe is 25 µg/ml and SO_4 is 1000 µg/ml.

A family of curves calculated from experimental data which shows the relationship of iron to cell numbers at six different pH values with sulfate level constant at 1000 µg/ml is presented in Figure 20. An attempt to show the three dimensional relationships among pH, iron concentration and numbers of Flavobacterium is illustrated in Figure 21. A series of similar drawings could be made for all possible combinations of three variables. The influence

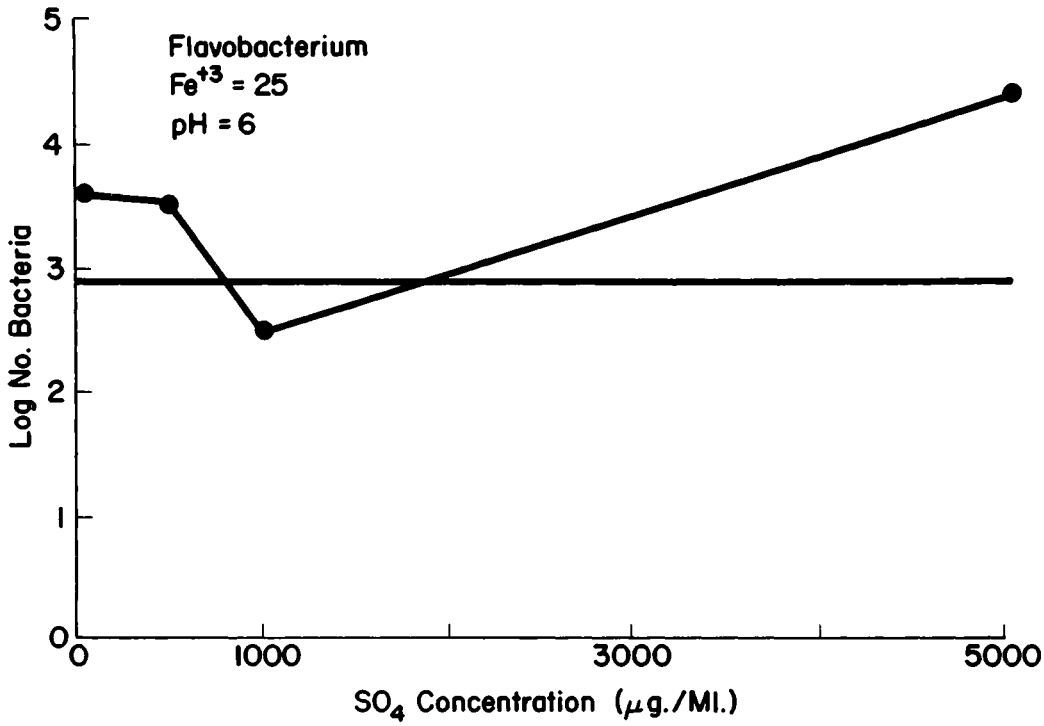

Fig 18

Plot of cell numbers vs dissolved sulfate concentration under the conditions specified.

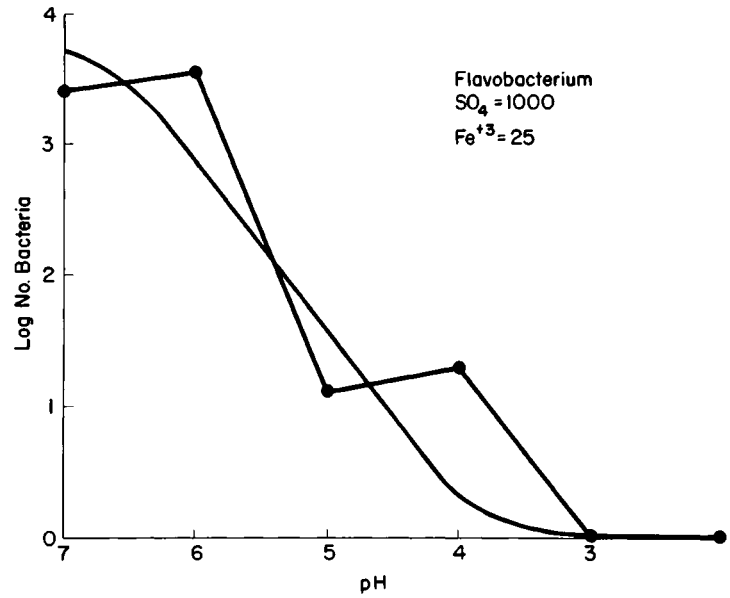

Fig 19

Plot of cell numbers vs pH under the conditions specified.

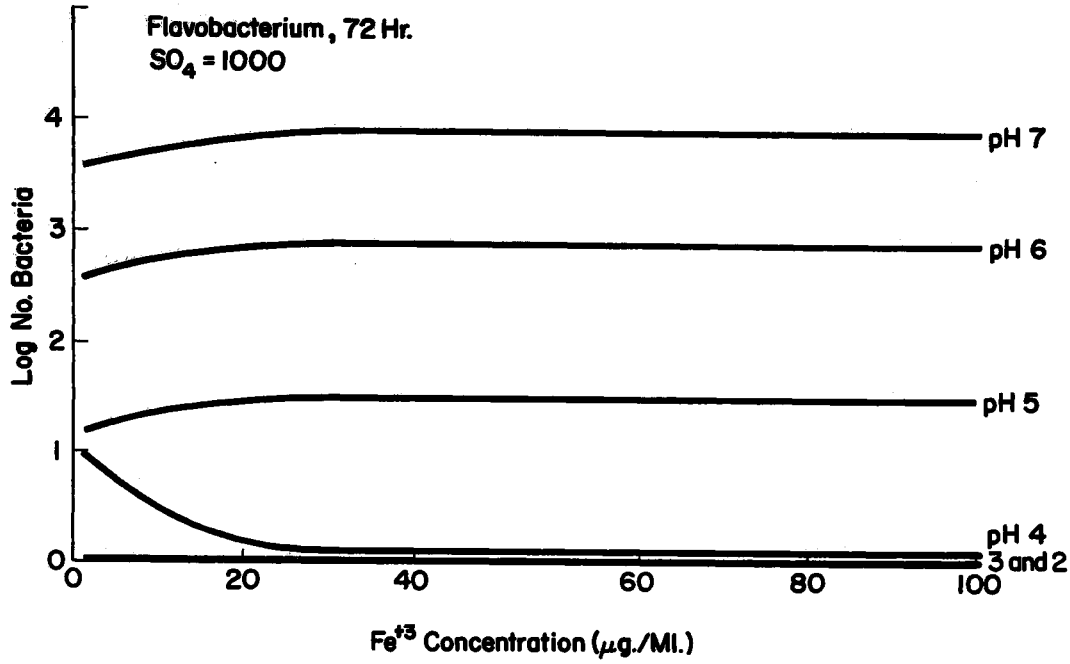

Fig 20

Family of curves showing plots of cell numbers vs iron concentration at six different pH valves under the conditions specified.

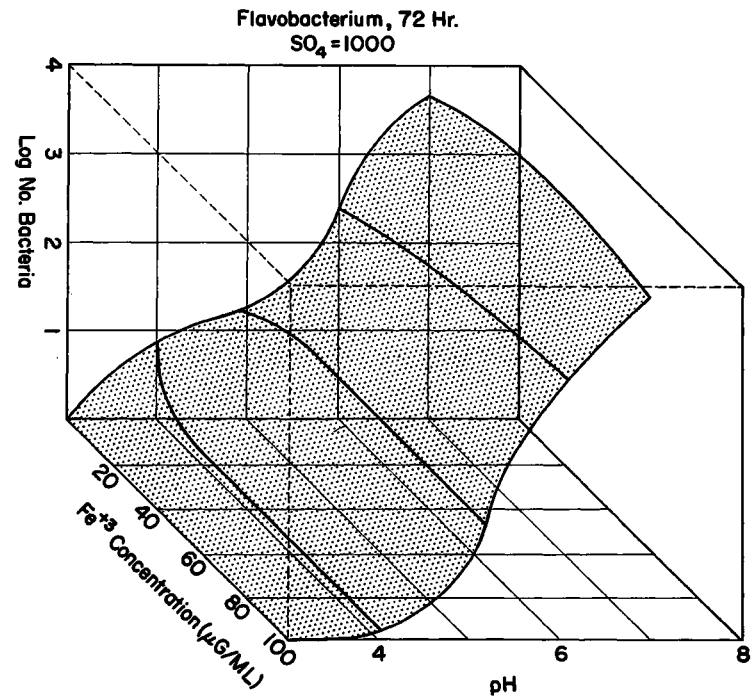

Fig 21

Plot showing number of bacteria vs iron concentration and pH under constant sulfate concentration. (1000μg/ml).

of the fourth variable (e.g. sulfate) on these particular relationships cannot be graphically included. That is, we cannot visualize four variables simultaneously.

Table 4 shows the mathematical models developed which represent the best fit curves of the experimental data. Various hypothesis concerning the response of organisms to all combinations of the variables studied can be tested statistically.

Values which can be predicted on the basis of the models would be expected to vary from the experimental values by less than 0.2 log unit. Predictions can therefore be made on the basis of the models within the frame work of our stated assumptions. Undoubtedly we have not considered all significant parameters involved in acidic streams. However, we do wish to stress the value of the approach toward solving complex biological problems.

In summary we are able to say that the test organisms should be able to grow in an acid stream when the pH is above 5.3, if iron levels are in the range 1-100 µg/ml and sulfate levels are in the range 50-500 µg/ml.

Growth of the bacteria increased with increasing iron content provided the pH was 5 or above. Therefore we probably should not overly concern ourselves with the iron content of waters from a biological viewpoint until the concentrations are considerably in excess of 100 µg/ml. This comment of course does not relate to considerations such as corrosion and other non biological situations.

In general we recognize complex relationships between sulfate and pH,

TABLE 4 Model Representing the Best Fit Curves of the Data for Three Different Bacteria

PSEUDOMONAS M1

$$\text{LOG BACT.} = 32 - 21(\text{pH}) + 4.4(\text{pH})^2 - 0.26(\text{pH})^3 + 0.54\ \text{LOG}(Fe^{+3}) - 0.014(\text{pH})^2\ \text{LOG}(SO_4^{=})$$

PSEUDOMONAS 229

$$\text{LOG BACT.} = 8.2 - 5.1(\text{pH}) + 1.3(\text{pH})^2 - 0.074(\text{pH})^3 - 0.64\ \text{LOG}(SO_4^{=}) + 0.21\ \text{LOG}(Fe^{+3}) \frac{-(\text{pH})}{\text{LOG}(SO_4^{=})}$$

FLAVOBACTERIUM M2

$$\text{LOG BACT.} = 6.4 - 5.5(\text{pH}) + 1.4(\text{pH})^2 - 0.084(\text{pH})^3 + 0.12\ \text{LOG}(Fe^{+3})$$

That is, sulfate influences growth of the organisms but not in a linear manner The variable of paramount importance appears to be H^+ , with some concern for sulfate. We believe, based upon the data and reasoning explained in this paper, that we should continue to emphasize our efforts to remove acidity and sulfate via metabolic activities of the sulfate reducing microbes as an abatement method. Methods of prevention should also be highly emphasized.

One might anticipate response to our generalizations by biologists who are interested in the welfare of organisms in streams other than bacteria. Microorganisms, once growing, will excrete metabolic byproducts into the environs which have buffering capacity. They also serve as nutrients for other organisms. The net result over a period of time is to re-establish some sort of biological equilibrium which then includes higher life forms. We also wish to emphasize that this study pertains to effects of acid water on the heterotrophic microflora of "normal" streams. Mrs. Macmillan in our laboratory has evidence that a high population of heterotrophic aerobes are also indigenous to certain highly acid water (pH 2.5). This population of microorganisms represent an ecosystem entirely different from the ecology of "normal" streams which we have been describing. The function of these microbes will have to be discussed at a later time.

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IV AEROBIC HETEROTROPHS INDIGENOUS TO pH 2.8 MINE WATER

This study represents another aspect of a continuing investigation of ecological relationships among microorganisms in acid mine effluents. We first reported studies of the change in the ecology of a neutral, uncontaminated stream upon contamination by acid runoff (Chapter I). A systems analysis for the purpose of differentiating a "healthy" from an acid-contaminated stream using microbiological criteria has also been reported (Chapter II). That study revealed the relative influences of iron, sulfate, and hydrogen ions on the normal heterotrophic microflora of a stream.

The present report concerns the ecology of the acid effluent from an abondoned drift mine which is located in Southeast Ohio near Lake Hope State Park. Figure [22] is a photograph of the mine entrance. The water pools to a depth of approximately eighteen inches at the mine opening and forms a shallow but constantly flowing stream away from the mine. The temperature of the runoff ranged between 7.5 and 10 degrees centigrade over a two year period.

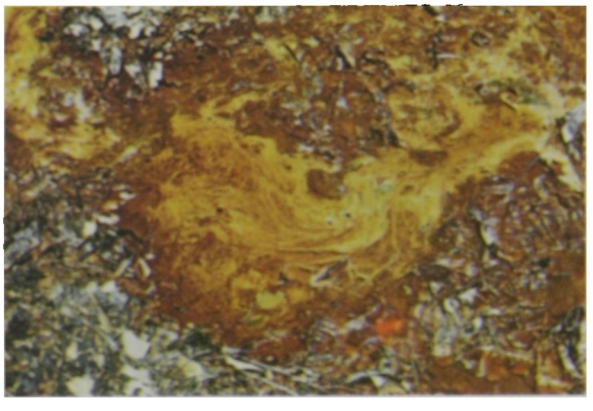
Table 5 shows a representative analysis of the effluent from this mine. The average pH is 2.73 with a range from 2.5 to 2.9. The levels of sulfate and hydrogen ions were in excess of those previously determined by systems analysis to be inhibitory to normal aquatic heterotrophs. Calcium, magnesium, and aluminum were also present in relatively high concentrations. Cations were analyzed by atomic adsorption spectroscopy (Perkin Elmer model No. 21). All other analyses comply to Standard Methods for the

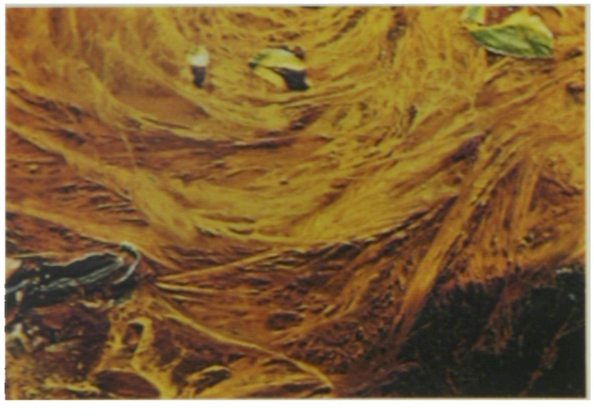
Figure 22. Photograph of Ohio number 47 Mine entrance showing colored acid discharge (pH 2.8).

Figure 23. Photograph of water immediately after discharge from mine opening showing gelatinous streamer growth.

Figure 24. Close up photograph showing detail of "streamers" presented in Figure 23.

Figure 25. Photograph showing Henrici slides in stream at point of discharge from mine. Slides and apparatus are overgrown with streamers. The dark shadow is cast by the mine entrance.





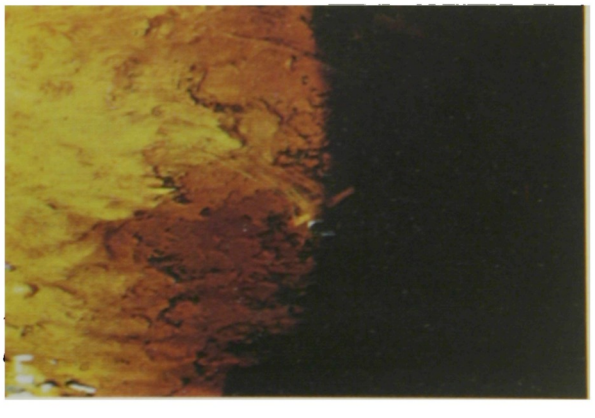


TABLE 5 REPRESENTATIVE ANALYSIS OF OHIO MINE #47 WATER

pH	2.73
Eh	520 mv
SPECIFIC CONDUCTANCE	4,680 umhos/cm
IRON	379 ppm
CALCIUM	248 ppm
MAGNESIUM	245 ppm
ALUMINUM	127 ppm
NICKEL	1.5 ppm
COPPER	0.3 ppm
MANGANESE	14.0 ppm
SODIUM	32.0 ppm
POTASSIUM	1.6 ppm
SULFATE	2,890 ppm
CHLORIDE	7 ppm
NITRATE	0.0 ppm
PHOSPHATE	0.5 ppm
TOTAL SOLIDS	7,020 mg/l
VOLATILE SOLIDS	1,183 mg/l

Examination of water and Wastewater (1).

Figure 23is a top view into the mine runoff from a distance of approximately four feet. Immediately obvious is a mass of gelatinous cream-colored streamers attached to leaves and other surfaces in the stream bed. Note the position of the two small leaves as a reference point for the next photograph (Figure 24)which is a close-up of the streamers. Such formations were first reported in the literature by Lackey (3), and were described as common in surface waters polluted by acid mine drainage. Since that time they have been referred to as "bacterial streamers" but, to our knowledge, no further characterization has been reported.

Most probable numbers of autotrophic bacteria were determined in tube cultures of 9K medium of Silverman and Lundgren (5). The MPN of iron oxidizers alone was found to be 9.2×10^5 per hundred mls. However, the observed streamers are not typical of the growth of autotrophic bacteria.

Preliminary attempts to isolate heretotrophs from the stream using standard microbiological methods were unsuccessful. We therefore employed the submerged slide technique of Henrici and Johnson (2) in an attempt to observe heterotrophic growth. This technique involves the concentration of organic materials from the environment on the surface of glass slides.

Clean, glass microscope slides were inserted in a holder constructed such that two slides would be suspended in a fixed position approximately 3 inches below the surface of the water. The entire assembly was sterilized by autoclaving prior to positioning in the pooled effluent at the mine entrance.

Figure 25 is a photograph of the assembly after 2 weeks submersion. The division in the photograph is a shadow cast by the mine entrance. Streamers are apparent and the slides are overgrown and unfit for microscopic observation. A time sequence study then followed. A series of sterile slides were placed in the effluent and removed after 1, 2, 3, 5 and 7 days. The slides were transported to the laboratory in screw cap Coplin staining jars containing distilled water and glutaraldehyde as a fixative. The following sequence of photographs are phase-contrast micrographs of wet mounts of microscope slides after staining with 0.03 per cent crystal violet.

The first of the series (Fig. 26) reveals a collection of small rods or diplo-rods at 24 hours. The refractile bodies are precipitated iron.

After 48 hours, the attachment of a morphologically distinct slender rod can be seen (Fig. 27).

After 3 days mirco-colonies of cells of at least two morphologically different types are apparent (Fig. 28).

Figure 29 shows increased growth after 5 days and a larger rod which appears to form filaments.

Between 5 and 7 days the system flourishes as is indicated in Fig. 30 which was taken after submersion for one week. The Large filamentous form is predominant but a mirco-colony of a smaller slender rod is apparent from the center to lower right of the photograph. Many of the very refractile bodies are iron precipitate, however, spores were also observed in the large rod.

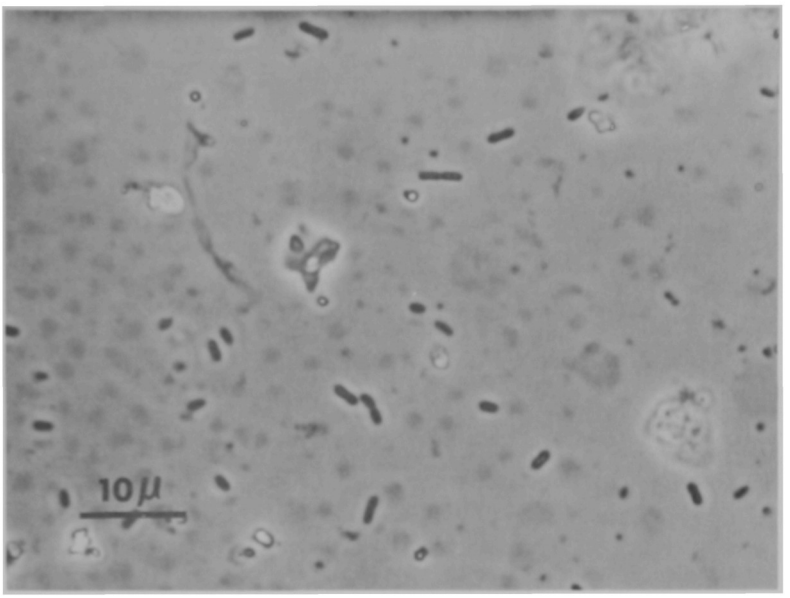


Fig 26

Phase contrast photomicrograph of glass slide immersed in pH 2.8 mine water for 24 hours, showing bacteria and inorganic particles.

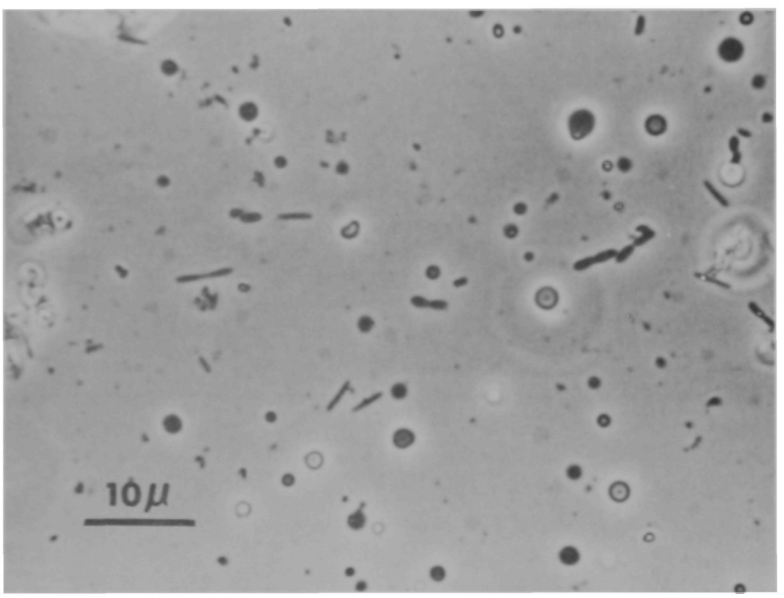


Fig 27

Phase contrast photomicrograph of glass slide immersed in pH 2.8 acid mine water for 48 hours, showing additional bacteria attached to the slide.

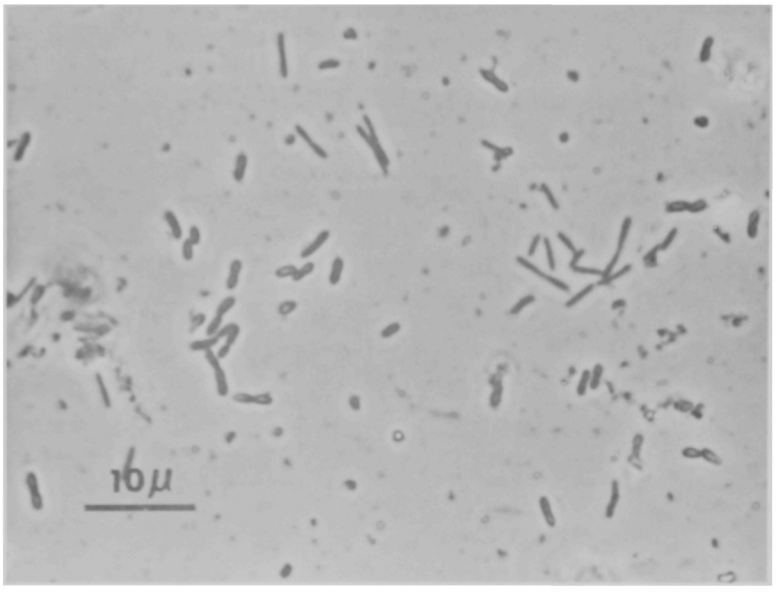


Fig 28

Phase contrast photomicrograph of glass slide immersed in pH 2.8 acid mine water for 3 days.

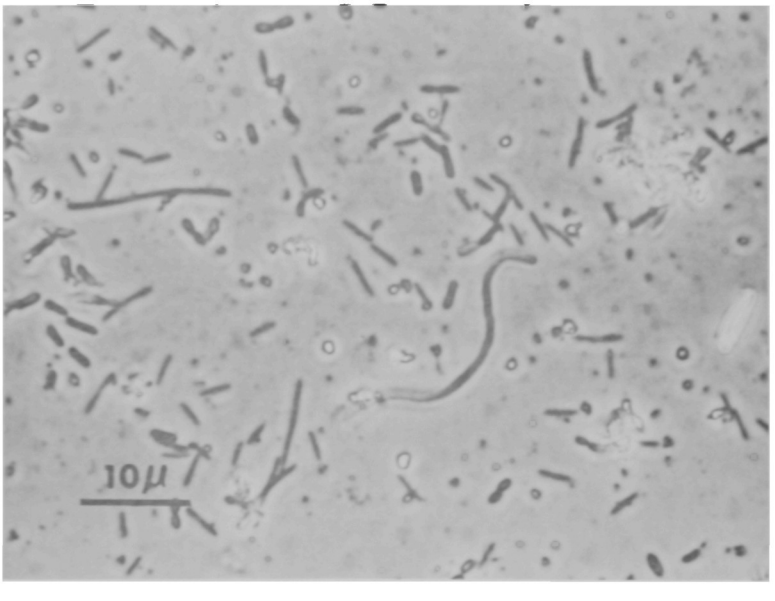


Fig 29

Phase contrast photomicrograph of glass slide immersed in pH 2.8 acid mine water for 5 days.

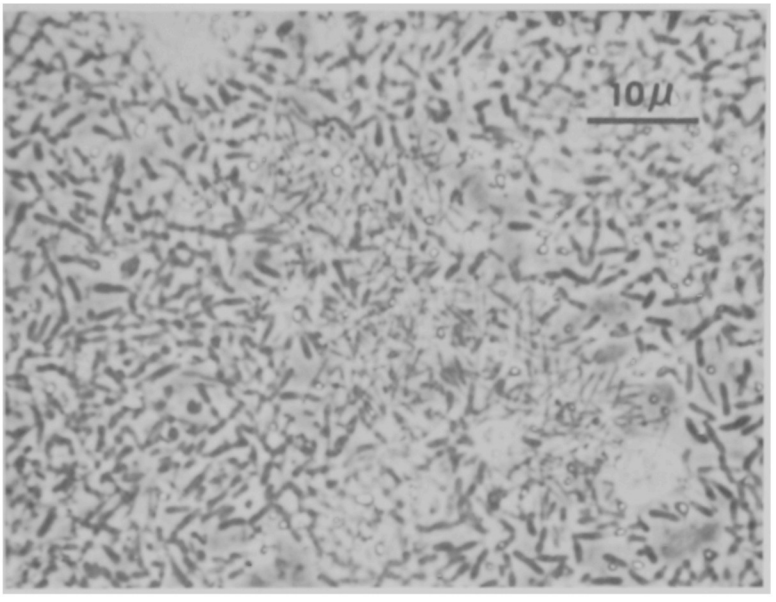


Fig 30

Phase contrast photomicrograph of glass slide immersed in pH 2.8 acid mine water for 7 days showing micro-colonies of bacteria of different types developing on the glass surface.

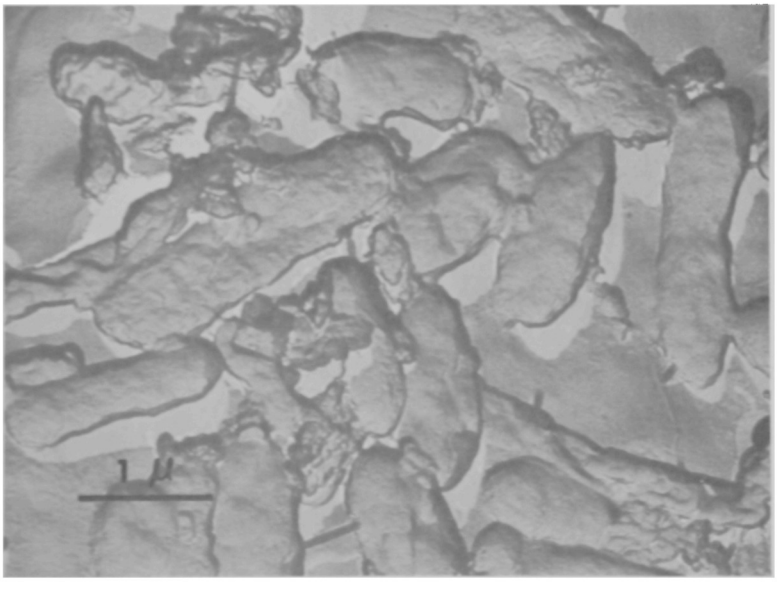


Fig 31

Electron micrograph of a preshadowed carbon replica of the surface of the glass slide shown in Fig 30. Various types of bacterial cells can be observed some of which have interconnecting strands of exocellular polymer, which is presumed to be the slime material shown in Figs 23, 24, and 25.

Figure 31 is an electron micrograph of a preshadowed, single stage, carbon replica which was prepared directly from streamers removed aseptically from the acid effluent. The gelatinous mass was broken by gentle aggitation on a vortex mixer and the resultant flocs were fixed overnight in an equal volume of glutaraldehyde solution before replication. The micrograph indicates that the streamers are composed of a mixture of rod-shaped bacteria. Apparent "bridges" between cells probably represent strands of exocellular "slime" which facilitates the agglomeration of cells and the formation of fixed macroscopic streamers in the flowing mine water.

By employing a medium containing an extract of precipitate and leaves from the stream bed, which we refer to as soil extract, and by utilizing Ion Agar 2 (Oxoid Co.) we have been able to isolate at least seven different aerobic, heterotrophic bacterial rods from the streamers. For this purpose water samples were collected according to Standard Methods (1). One-half milliliter of sample was aseptically spread on the agar surface and incubated from 3 to 7 days at 20 degrees centigrade. A slight stimulatory effect of the soil extract was demonstrated by a total count of 500 per ml. as compared to 400 per ml. on the same medium lacking soil extract. The medium containing soil extract plus purified agar yielded a count 300 per cent greater than that obtained on standard Plate Count Agar (DIFCO).

Table 6 summarizes some characteristics of the isolated organisms which include one yeast, two Gram positive rods, and five Gram negative rods. Two of the isloates requiré soil extract for growth and three appear to be inhibited by unpurified agar. None ferment the sugars examined,

TABLE 6 HETEROTROPHS FROM pH 2.8 MINE WATER

ISOLATE	A	B	C	D	E	F	G	H
GRAM REACTION	+	YEAST	-	-	+	-	-	
TGE+YE+SE	+	+	+	+	+	+	+	+
TGE+YE	+	+	+	N.G.	+	N.G.	+	+
PCA	+	N.G.	+	N.G.	+	N.G.	+	+
GLUCOSE	-	-*	-	-*	-	-*	-*	-
SUCROSE	-	-	-	-	-	-	-	-
LACTOSE	-	-	-	-	-	-	-	-
MANNITOL	-	-	-	-	-	-	-	-
GELATIN	-	N.G.	-	N.G.	-	N.G.	N.G.	+
NITRATE	-	N.G.	-	GAS	+	-	GAS	-
CITRATE	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G
PIGMENT	-	-	-	-	-	-	-	-

+ = GROWTH

- = GROWTH WITH NEGATIVE REACTION

* = VERY LITTLE GROWTH

N.G. = NO GROWTH

one hydrolyzes gelatin, two reduce nitrate to nitrogen gas, and one reduces nitrate to nitrite.

Isolate A (Table 6) is a large, Gram positive, filamentous bacterial rod which forms spores as observed by phase-contrast microscopy and confirmed by resistance to heat shcoking at 80° C for 15 minutes. When incubated on an agar surface at 10 degrees centigrade this organism forms copious amounts of exocellular slime. This could facilitate streamer formation in the runoff. This also appears to be the filamentous rod which overgrows the glass slide when submerged for 7 days in the mine water although no conclusive experiments are available to verify this contention.

We have also observed and identified an algal cell which forms a green coating on submerged surfaces in dim light at the mine entrance. Figure 32 is a photomicrograph of the cell which has been identified as Euglena mutabilis. This cell migrates by creeping and is a characteristic species of acid habitats (4). The ecological significance of this organism, as well as that of the bacteria, is being further investigated.

It is apparent that acid streams which are generally recognized as environments too extreme to support a significant amount of biological activity are indeed capable of sustaining a mixed microflora. Although quantitative estimates of microbial growth cannot be made because of the sessile nature of the slime matrix, the observed amount of biomass would suggest presence of a significant amount of biological activity in what has been considered an extreme evironment. It also suggests that such streams

Fig 32

Photograph of the alga Euglena Mutabilis which forms a dense green film in the acid water inside the mine entrance.

would recover rapidly toward a more neutral pH if the continuous addition of acid were stopped because the net result of heterotrophic metabolism would not approach the acidity of mineral acids.

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V. A MICROBIAL DISSIMILATORY SULFUR CYCLE[1]

Autotrophic bacteria in the Thiobacillus-Ferrobacillus group are responsible for the enzymic oxidation of ferrous sulfide minerals (e.g. pyrite and marcasite) which are often found associated with coal in nature (3). The net result of microbial oxidation of pyritic minerals is an accumulation of ferric, sulfate and hydrogen ions. Drainage water in regions where such minerals are mined become highly acidic and contain relatively high concentrations of iron, sulfate and other ions. A marked alteration of the microbial ecology can be observed under these conditions and certain aspects of this ecosystem have been described (12). The inhibitory effects of acid mine water on heterotrophic bacteria of neutral streams was reported to be due to hydrogen ions and to a lesser extent, sulfate ions but not to iron ions (6).

This report considers the metabolic activities of microorganisms involved in the reduction of iron and sulfate ions, and the neutralization of acid in naturally occurring acidic mine water.

Figure 33 is a schematic representation of the acidic stream studied. The stream flow has been impeded by a dam composed primarily of wood dust which is a waste product from a small log cutting mill. The retarded flow of water results in a pond behind the dam and is hereafter referred to as the Upper Pond. Uneven terrain downstream from the dam resulted in formation of the Lower Pond. Water was examined at the six different locations shown in Fig. Although samples from all locations were required for the evaluation, the greatest emphasis is given to samples from the Upper and Lower Ponds.

1. Published in Journal of Bacteriology. 1969 (in press).

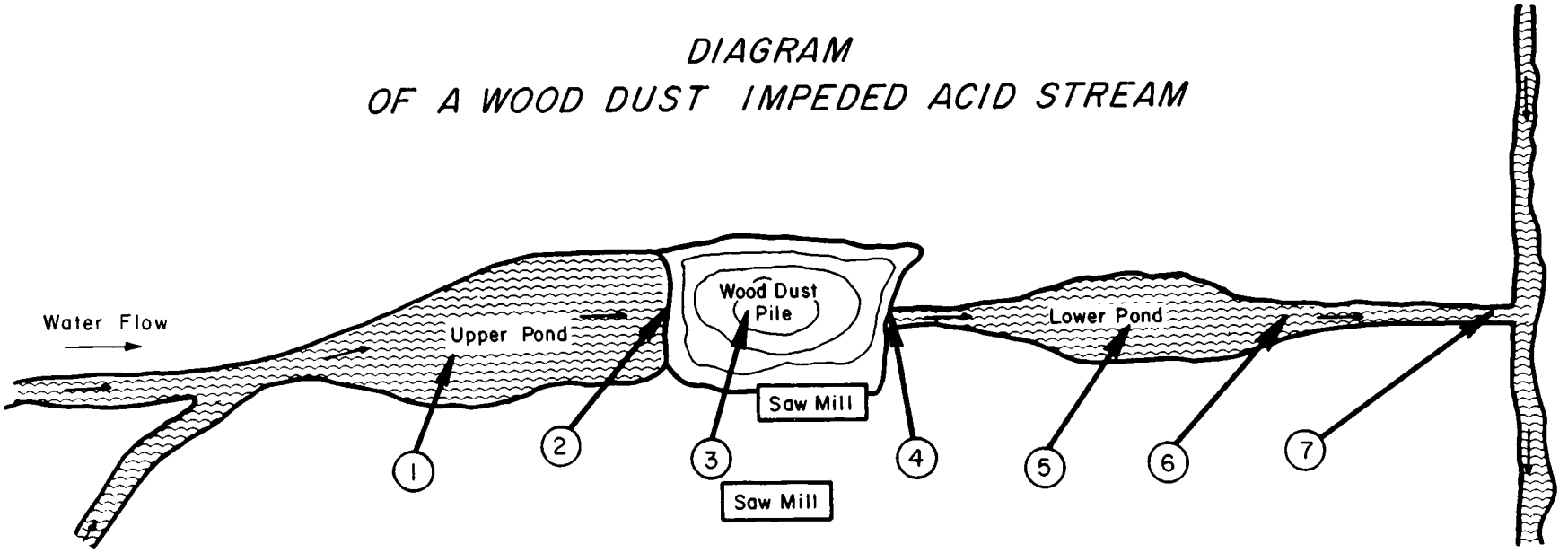

Fig 33

Schematic outline of an acid stream which is impeded by a dam composed of wood dust; showing the upper and lower ponds and sample locations.

MATERIALS AND METHODS

Samples Water samples were taken in the field in sterile 8 oz bottles and held on ice in a styrofoam cabinet until they reached the laboratory and were then refrigerated at 8 C. All water samples were plated on bacteriological culture media within 24 hr after they were taken from the stream.

Chemical Determinations Total dissolved iron was measured colorimetrically by the phenanthroline method according to the procedure described for a Hach Field Kit (Hach Chemical Co., Des Moines, Iowa).

Sulfate was determined turbidimetrically with $BaSO_4$ precipitate as described by the Hach procedure, and pH was determined with a Beckman pH meter.

H_2S was semi-quantitatively determined by the Hach procedure which involved comparison of the amount of black PbS precipitate formed on a lead acetate impregnated filter paper with papers blackened by a known concentration of H_2S. This procedure was sensitive to 0.1 μg H_2S/ml of water.

Pond water was analyzed directly for aldehydes by the Tollens method (8) and after 20X and 40X concentration by evaporation at 100 C.

Chromatography of soluble sugars in wood dust extracts Extracts were prepared by adding 200 ml of distilled water to 100 g wood dust which was obtained from either the top 6 in. (fresh wood dust) or from a depth of 3 ft into the pile (partially decomposed wood dust). The mixtures were allowed to stand at ambient temp. for 2 hr with frequent shaking. Large particulates were then removed by filtration through Whatman no. 1 paper. The resulting

filtrates were refiltered through sterile 0.45 μ pore size membranes (Millipore Filter Corp., Bedford Mass.).

One hundred ml of each extract was vacuum evaporated to dryness over KOH pellets at 22 ± 2C. The dried residues were prepared for chromatography by resuspending in 1.0 ml of distilled water. One hundredth ml of each solution was applied separately to Whatman no. 1 paper which had been previously spotted with glucose, xylose, cellobiose, and galactose marker solutions. Descending chromatograms were developed in isopropanol: water (1:4) at room temperature. After development for 24 to 27 hr, sugars were detected by the Trevelyan silver nitrate method (11). A 40X concentration of Lower Pond water was also chromatographed in butanol: acetic acid: water (40:10:22). The concentrated sample was dialyzed against distilled water (8 C) for 12 hr and chromatographed in the same solvent system.

Mixed Culture Flask Systems Four hundred g wood dust and 1 liter of water were added to 2-liter Erlenmeyer flasks in the following combinations of wood dust and water: acid mine water plus partially decomposed wood dust, acid mine water plus fresh wood dust, distilled water plus partially decomposed wood dust, distilled water plus fresh wood dust. Separate flasks containing each of the four combinations were incubated at 22 ± 2C, 37C, and 50C respectively. Supernatants from flasks containing acid mine water were routinely assayed for iron, sulfate, and pH as described above. All flasks were assayed for carbohydrates by the anthrone method of Steinecher and Rheins (10) and by the Nelson reducing sugar test (2).

Media and Growth Conditions Portions (1 ml or appropriate dilutions of each sample were pour plated on Tryptone Glucose Extract (TGE) Agar (Difco), which was supplemented with 0.5 g yeast extract per liter (TGYE), and on Sabouraud's Dextrose (SD) Agar (Difco). The cultures were incubated at 25 ± 2 C in the air for 3 days.

Anaerobic microorganisms, other than Desulfovibrio, were determined by adding 1.0 ml of Thioglycollate Medium (Difco). The reciprocal of the highest dilution that showed growth after 7 days at 25± 2 C , as determined by appearance of turbidity in the anaerobic zone of the tubes, was taken as the number of organisms.

Sulfate-reducing bacteria were enumerated using a standard three tube most probable number (MPN) method (1) , according to the tube culture technique described by Postgate (7) with Desulfovibrio desulfuricans medium No. 3. Positive tubes were black after incubation at 25 ± 2 C for 6 to 21 days.

Cellulose digesting microorganisms were isolated and cultured in the cellulose enrichment medium of McBee (5). Cellulose was obtained from rope cotten which was hydrolyzed in conc. HCl for 48 hr at 25 ± 2 C, washed 7 times with distilled water, and ground to a paste with a mortar and pestle. Plates were incubated at 25 ± 2 C for 14 days. Cellulose digesters were identified by the appearance of a clear zone around the colony.

Iron-oxidizing chemoautotrophic bacteria were counted by a five tube MPN technique (1) in the salts medium of Silverman and Lundgren (9).

Positive tubes were determined after 15 days at 25 ± 2 C by presence of a dark red- brown precipitate. Uninoculated control tubes and those in which growth did not occur contained a light yellow-brown precipitate at the end of the incubation period due to autooxidation of ferrous iron.

Sulfur-oxidizing chemoautotrophic bacteria were enumerated in the same manner as were the iron oxidizers. Elemental sulfur (0.1 g per 10 ml) replaced the $FeSO_4 \cdot 7H_2O$ in the culture medium, and positive tubes were taken as those in which sufficient acid was produced to cause 5 drops of a 1% thymol blue solution to turn red (red pH 1.2 to yellow 2.8). Control tubes and negative growth tubes gave a yellow indicator reaction.

Aerobic heterotrophic and facultative heterotrophic isolates other than Streptomyces were examined for ability to ferment or assimilate glucose, lactose, adonitol, arabinose, dulcitol, galactose, inositol, inulin, levulose, maltose, sorbitol, trehalose, and xylose. Glucose and lactose utilization was determined in tubes of Purple Broth Base (PBB, Difco) which contained 1.0% (w/v) sugar. All other carbohydrates were added as sterile differential discs (Difco) to plates of PBB containing 1.5% agar, which had been spread with 0.1 ml of a 24 hr nutrient broth culture of the isolate. All cultures were incubated 25 ± 2 C for 48 hr. A color change of purple to yellow (brom cresol purple, yellow pH 5.2 to purple pH 6.8) was taken as an indication of fermentation. Enhancement of growth around the disk was considered an indication of oxidative utilization.

Nitrate reduction was determined according to the method described in

the Difco Manual (4). Cultures were incubated at 25 ± 2 C for 18 hr. One tenth g of zinc dust was added to each negative tube and the presence of a pink color assured that the nitrate had not been reduced further than nitrate, thereby causing a false negative observation.

Gelatin liquefaction was determined by the method described in the Difco Manual (4) after incubation at 25 ± 2 C for 5 days.

Streptomyces species isolated from wood dust were tested for their ability to ferment cellulose, glucose, and xylose at 22 ± 2 C, 37 C, and 50 C. Cells were inoculated into PBB containing 1% (w/v) glucose, xylose, or cellulose (Whatman no. 1 powder) and incubated for 48 hr at the respective temperatures.

RESULTS

Table 7 is a compilation of average data obtained from samples taken from the Upper and Lower ponds over a one year period. Each average represents a minimum of four determinations. The sulfate-reducing bacteria from the lower pond represent two different types. These have been tentatively identified as a Desulfovibrio and a Desulfotomaculum species.

Data obtained from samples taken on the same day are summarized in Table 8. The heterotrophic aerobes listed in Table 7 consisted of 10 different yeasts and 12 different bacteria.

Ten different yeasts were isolated from the Upper and Lower Ponds and were examined for sugar fermenting capacity because of their potential

TABLE 7. Chemical and biological changes which occurred in acid mine water upon passage through wood dust. Bold values are averages calculated from a minimum of four readings. Ranges of values are given in parentheses beneath the average values.

	Sample Location in Fig. 1	
	1 Upper Pond	2 Lower Pond
pH	**2.84** (3.90-2.40)	**3.38** (4.85-2.70)
SO_4 Concentration uM. ml	**8.765** (5.205-12.492)	**6.100** (3.227-10.306)
Total Fe Conc. uM/ml	**1.067** (0.788-1.325)	**0.313** (0.064-0.681)
S oxidizing bacteria MPN/100ml	**9,580** (130-33,000)	**1,820,000** (23,000-7,000,000)
Fe oxidizing bacteria MPN/100 ml	**9,520** (490-33,000)	**426,000** (33,000-1,400,000)
Anaerobes/ml Thioglycollate	**2.5** (0-10)	**528** (10-1,000)
$SO_4^=$ Reducers MPN/100 ml	**0**	**876** (0-2,400)
Heterotrophic Aerobes/ml		
SD Agar	**15.4** (2.44)	**82,100** (49-290,000)
TGYE Agar	**47.4** (2.5-110)	**350,000** (470-1,700,000)

Table 8. Chemical, biological and temperature differences in samples collected on the same date.

Sample Location (Fig. 1)	Air Temp. °C	Sample Temp. °C	pH	$SO_4^=$ Conc. uM/ml	Total Fe Conc. uM/ml	S Oxidizers MPN/100ml	Fe Oxidizers MPN/100 ml	$SO_4^=$ Reducers MPN/100 ml
1	-6	6	3.1	10.098	1.325	1.3×10^2	4.9×10^2	0
2	-6	6	2.8	9.890	1.468	1.1×10^2	8.0×10^1	0
3	-6	33	-	-	-	-	-	-
4	-6	13	4.3	3,825	0.681	1.0×10^7	4.9×10^5	7.0×10^2
5	-6	6	3.4 4.0*	3.825 4.268*	0.663 0.734*	7.0×10^6* 7.9×10^6	1.4×10^6 1.0×10^7*	1.4×10^3 1.9×10^3 *
6	-6	6	3.35	4.268	0.573	2.2×10^4	2.1×10^5	2.0×10^2
7	-6	6	3.2	5.309	0.645	4.9×10^4	4.6×10^5	

* Samples taken at bottom-water interface.

production of alcohols and organic acids which could serve as nutrients for the dissimilatory sulfate-reducing bacteria found in both the wood dust pile and the Lower Pond. Sugar utilization capabilities of the yeast isolates are summarized in Table 9 . Three of the isolates have tentatively been characterized as strains of Rhodotorula glutinis . None of the isolates fermented or utilized the following compounds: dulcitol, inositol, inulin, melibiose, rhamnose, salicin, sorbitol, and trehalose.

Four gram positive isolates tentatively classified as Bacillus species, were obtained from the Upper and Lower ponds. Table 10 summarizes some of the biochemical properties of these isolates. Additional sugar and sugar alcohol utilization tests were performed on isolates no. 1 and no. 4 as described in the Methods section. Isolate no. 1 also utilized maltose, salicin, sorbibitol, and trehalose. Isolate no. 4 fermented salicin with the production of acid. Neither no. 1 nor no. 4 had any activity on the remaining carbohydrates examined.

Seven physiological types of gram negative rod-shaped bacteria were also isolated from the Upper and Lower ponds. One of these types, representing 2 isolates, was tentatively classified as Aerobacter species. The remaining six types, comprising 25 isolates, were tentatively classified as Pseudomonas species. The majority of these isolates had little fermentative ability.

Microbiological examination of the wood dust pile yielded seven different Streptomyces isolates. A summary of the sugar utilization by these isolates after 48 hr incubation is shown in Table 11. In general, the isolates grew much

Table 9. Biochemical reactions[a] of 10 yeast isolates from an acidic ecosystem.

Isolate No.	Tentative Nomenclature	Sample Station	pH of Sample	Pigment	Adonitol	Arabinose	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Raffinose	Xylose	Nitrate Reduction
1	Rhodotorula glutinis	4	2.7	Red	-	-	A	-	+	+	-	-	A	-	-	-
2	Rhodotorula glutinis	1	2.8	Red	-	-	+	-	+	+	+	-	+	-	-	-
3	Rhodotorula glutinis	1	3.8	Red	+	-	+	+	+	+	+	-	+	-	I	+
4		4	3.9	None	-	-	+	-	A,G	+	-	-	-	-	-	-
5		4	3.6	None	-	-	+	+	A	+	+	+	+	-	I	-
6		4	2.7	None	-	-	-	-	+	+	-	-	-	-	-	-
7		4	3.9	None	-	-	+	+	+	+	+	+	+	-	I	+
8		1	2.8	None	-	-	+	-	+	+	-	-	+	-	-	+
9		1	3.5	None	-	-	-	-	+	+	-	-	-	-	-	+
10		1	3.2	None	-	+	+	+	+	+	+	-	+	+	-	-

[a]Reactions are indicated as follows: -, negative response; +, growth but no acid; A, acid produced; G, gas produced. None of the organisms utilized dulcitol, inositol, melibiose, rhamnose, salicin, sorbitol, or trehalose.

Table 10. Biochemical reactions[a] of 4 Bacillus isolates from an acid ecosystem.

Tentative Genus	Glucose	Lactose	Oxidase	Nitrate Reduction	Gelatin Liquefaction	Motility	Sample Station of Isolation (Fig. 1)
Bacillus No. 1	Acid	Acid	-	-	+	+	1, 2, 5
Bacillus No. 2	Acid	Acid	-	+	+	+	1, 2
Bacillus No. 3	Acid	-	-	+	+	+	1, 2
Bacillus No. 4	Acid	-	-	-	+	+	1, 2

[a]Reactions are indicated as follows: -, negative response; +, positive response.

Table 11. Utilization of glucose, cellulose and xylose by Stroptomyces isolates at three different temperatures.[a]

Isolate Number	Glucose			Cellulose			Xylose		
	22C	37C	50C	22C	37C	50C	22C	37C	50C
1	-	+	-	±	+	-	±	±	-
2	+	++	-	+	++	-	+	++	-
3	A	A	-	++	++	-	++	++	-
4	+	+	-	+	+	-	++	+	-
5	-	+	-	-	+	-	-	++	-
6	-	-	-	-	-	-	±	-	-
7	-	-	-	-	-	-	-	-	-

[a]Reactions are indicated as follows: -, negative response; ±, slight utilization; +, utilization: ++, greater utilization than +; A, acid produced.

better at 37 C than at either 50 C or 22 C. Isolates 6 and 7 had little activity on the substrates tested at any of the incubation temperatures employed.

One white rot type Basidiomycete was observed to have extensive mycelial growth in the top several inches of the wood dust pile. In addition, one cellulolytic yeast which appeared to be a Rhodotorula species and one anaerobic gram (+) bacillus, tentatively identified as Clostridium were isolated. Gram (-) rod shaped bacteria were also present in the wood dust. No attempt was made to isolate and identify these species, except one, which was similar to the Aerobacter species isolated from the Upper Pond.

Aldehydes were present in both Upper and Lower ponds (Tollens method) but intensity of reaction indicated semi-quantitatively that a greater concentration of aldehydes was present in the Lower pond.

Chromatographic examination of concentrated Lower Pond water indicated the presence of sugars. The components could not be resolved by the techniques employed, and much tailing occurred because of the high salt content of the water. Dialyzed concentrates did not contain sugars. Fresh wood dust extracts also contained sugars. Two components co-chromatographed with glucose and xylose markers. Partially degraded wood dust extracts did not contain these components. Both extracts contained inseparable components which did not migrate as far as the glucose, xylose, cellobiose, and galactose markers.

Figures 34 and 35 show the effect of temperature and wood dust

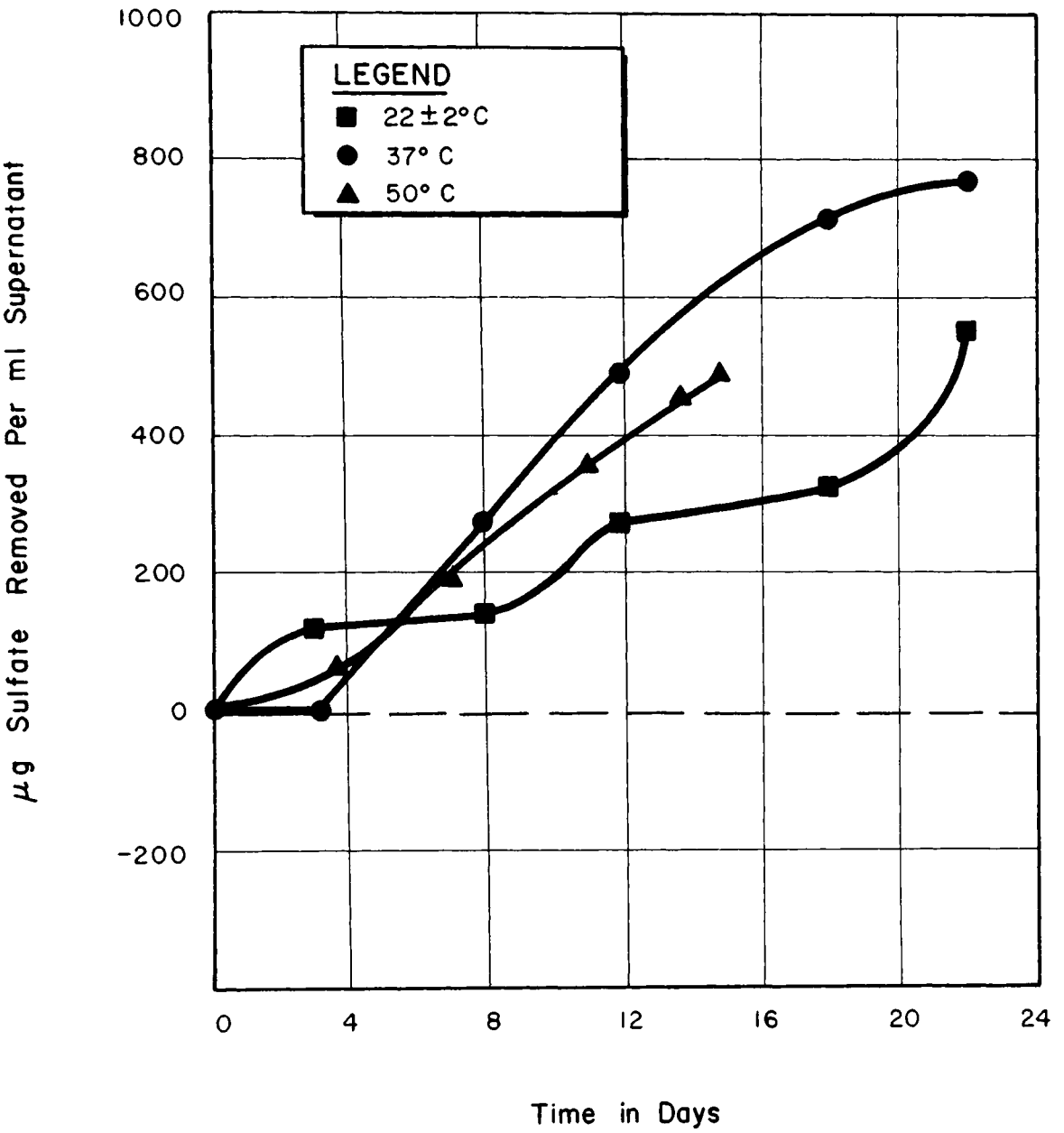

Fig 34

The influence of temperature on sulfate reduction in flasks containing a mixed microflora and partially decomposed wood dust.

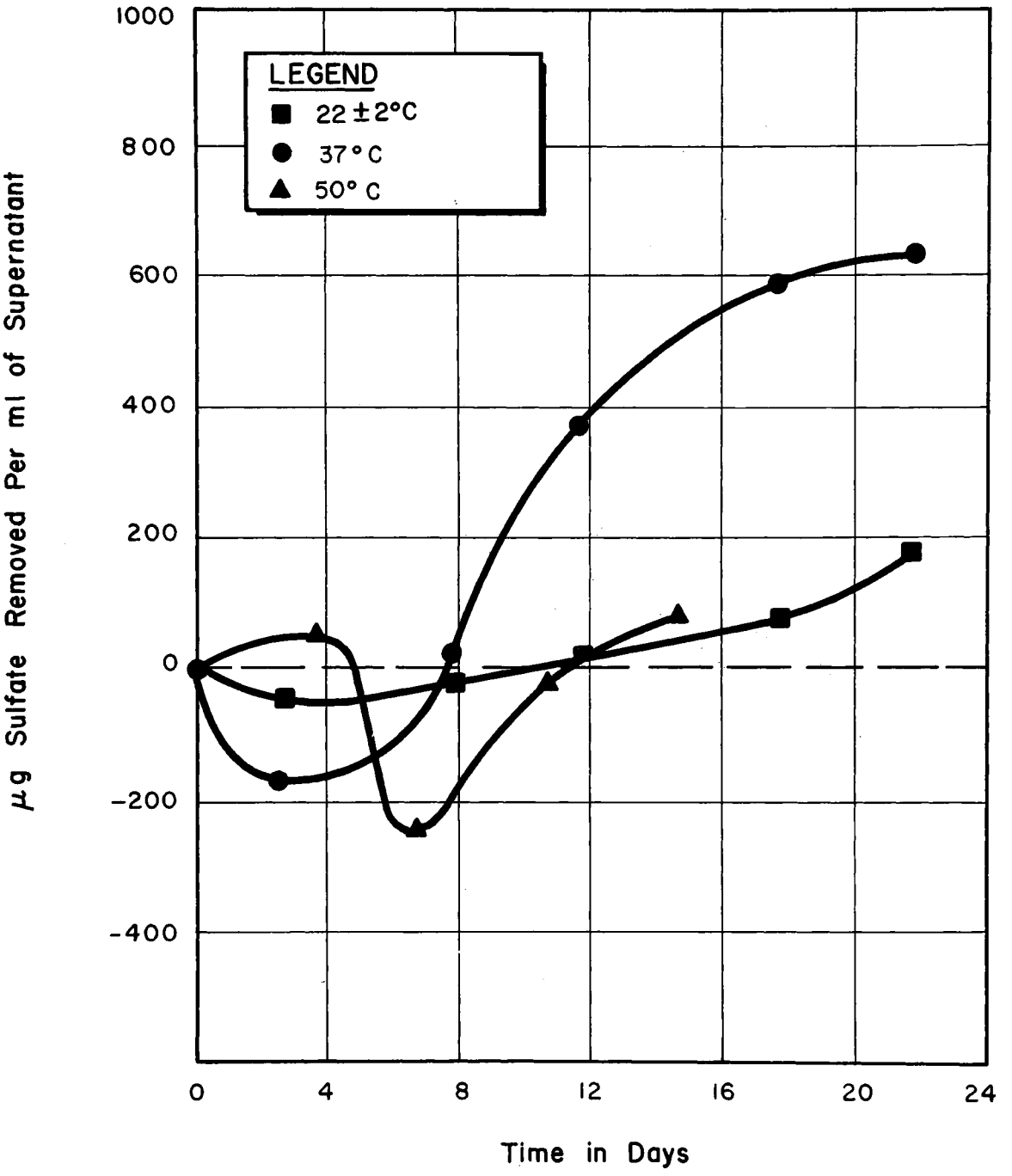

Fig 35

The influence of temperature on sulfate reduction in flasks containing a mixed microflora and fresh wood dust.

condition on sulfate reduction. Maximum sulfate reduction occurred in flasks containing partially degraded wood dust.

Figures 36 and 37 show the changes in pH in the same flask cultures. Note that the rise in pH correlates with the removal of sulfate.

Table 12 is a compilation of the anthrone and reducing sugar concentrations in culture supernatants which contained a mixed microflora taken from wood dust. Data from flask cultures in which distilled water was substituted for acid mine water are also included. The anthrone procedure measures total soluble sugars, providing that the monomeric units are able to form furfurals in the presence of concentrated sulfuric acid. In contrast to the anthrone procedure, the Nelson's test does not hydrolyze glucosidic linkages. Therefore, an increase in reducing power over the amount of anthrone measurable sugar is interpreted as a decrease in the average chain length of soluble cellulose polymers.

DISCUSSION

Water entering the Upper Pond was typical of acidic mine drainage as regards low pH, high iron and sulfate ion concentrations, numbers of iron and sulfur oxidizing autotrophic bacteria, and low numbers of heterotrophic bacteria, particularly anaerobes. No sulfate-reducing bacteria could be found in the Upper Pond. Four gram positive species were isolated from the Upper Pond and were tentatively identified as Bacillus species. This is of interest because we had previously reported that gram positive bacteria of

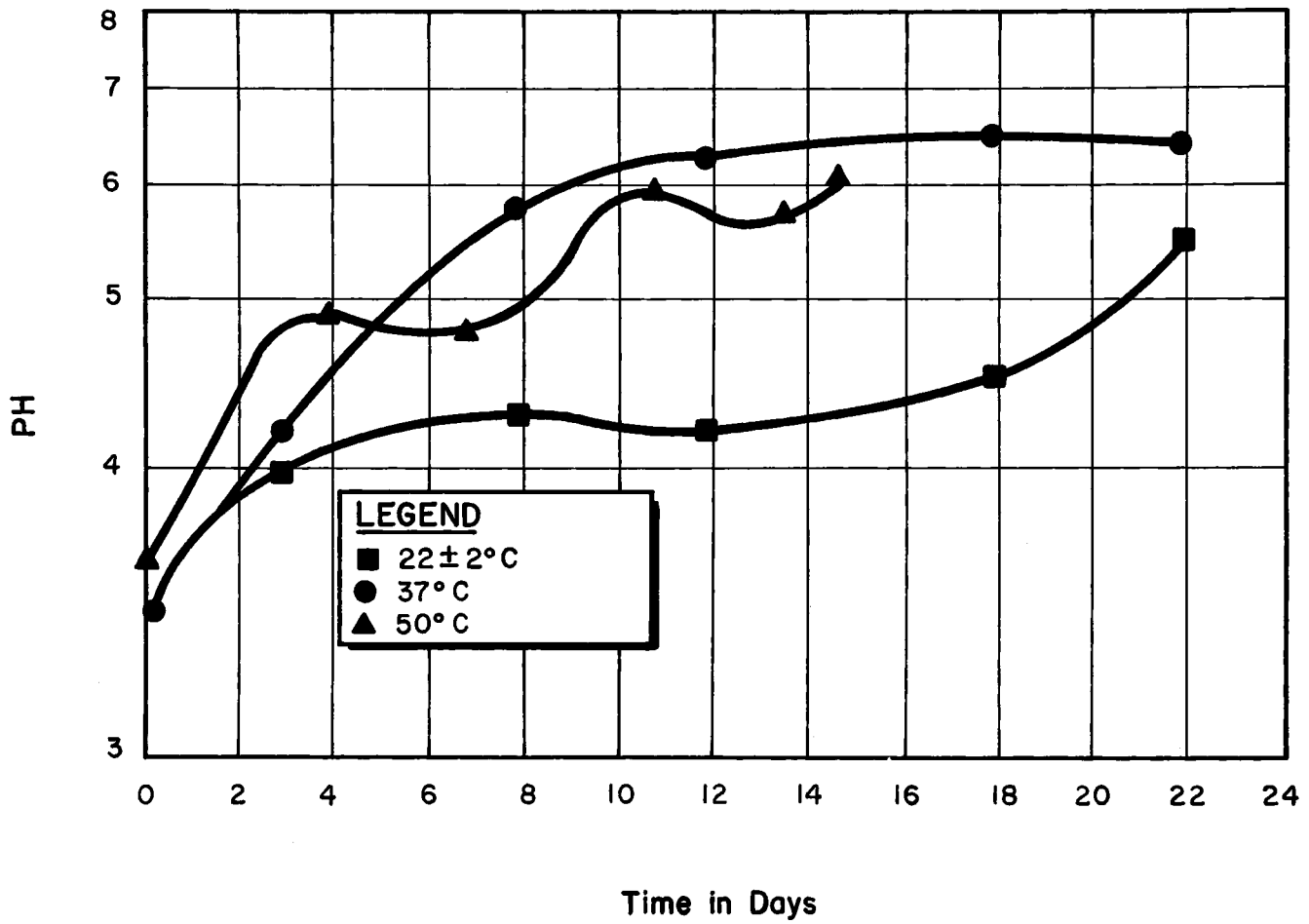

Fig 36

The influence of temperature on pH change in flasks containing a mixed microflora and partially decomposed wood dust.

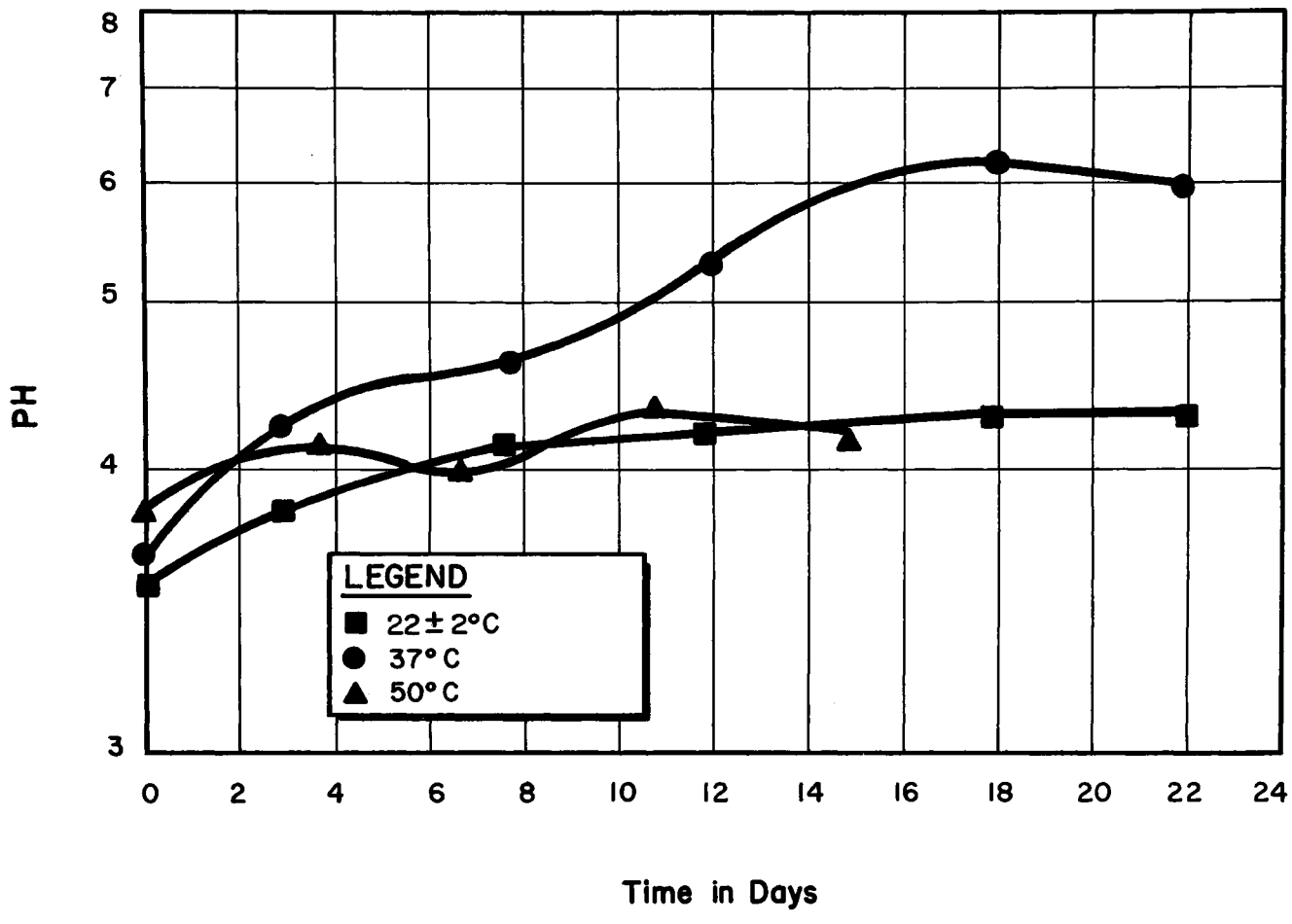

Fig 37

The influence of temperature on pH change in flasks containing a mixed microflora and fresh wood dust.

Table 12. Values showing change (ug/ml) in concentration of total and reducing sugars as result of mixed fermentation of wood dust at three different temperatures.

		22° C			37° C			50° C		
		O Time	14 Days	Net Gain	O Time	14 Days	Net Gain	O Time	14 Days	Net Gain
DISTILLED H_2O + FRESH WOOD DUST	A[1]	275	360	+85	275	215	-60	185	440	+225
	R[2]	70	250	+180	60	200	+140	175	350	+175
ACID MINE H_2O + FRESH WOOD DUST	A	190	240	+50	170	235	+65	130	460	+330
	R	45	130	+85	15	350	+335	95	275	+180
DISTILLED H_2O + PARTIALLY DEGRADED WOOD DUST	A	65	55	-10	65	55	-10	30	85	+55
	R	50	45	-5	40	50	+10	40	245	+205
ACID MINE H_2O + PARTIALLY DEGRADED WOOD DUST	A	45	50	+5	40	45	+5	5	95	+90
	R	0	5	+5	20	40	+20	10	235	+225

A^1 = Anthrone total soluble sugars

R^2 = Nelson's reducing sugars

neutral streams were extremely susceptible to acid mine water (12).

In contrast, the Lower Pond contained a vastly different microflora. Aerobic heterotrophic microorganisms were present in markedly higher numbers. The predominant aerobes or facultative aerobes were yeasts and higher fungi and 12 different physiological types of bacteria. Most were tentatively classified as Pseudomonas species. Higher plate counts on TGYE agar than on the more acid SD agar indicates that many of the organisms in the Lower Pond were acid tolerant and viable, but grew more favorably on the neutral TGYE growth medium. This suggests that they were entering the Lower Pond from the wood dust pile and were probably not growing in the Lower Pond.

A 200 fold increase in numbers of anaerobic bacteria was observed in the Lower Pond as compared to the Upper Pond. This is undoubtably due to organic materials from the wood dust which serve as nutrient sources, and the effect of these nutrients on lowering the O/R potential.

The increase in pH, and the decrease in iron and sulfate concentrations (Table 7) in the Lower Pond in comparison to the Upper Pond can only be attributed to the influence of the wood dust dam. This can be seen by comparing the chemical parameters at station 4, where the water leaves the wood dust, with the same parameters at station 5 (Table 8). Although some activity with respect to sulfate reduction occurs in the Lower Pond, the majority of the sulfate reduction process undoubtedly occurs in the wood dust. The discrepancy between chemical parameters at the surface and at the bottom of the Lower

Pond probably result from the ability of the chemical assays used to pick up precipitates as $FeSO_4$ in the pond bottom samples.

Stream flow rates which are influenced by environmental conditions caused fluctuations in measured parameters in the field. The parameters appeared to coincide with observed water conditions. For example, when the Lower Pond had a reddish color, the sulfate concentration and pH were nearly equal to the Upper Pond water. Under these conditions, low numbers of sulfate reducers were recovered. When the Lower Pond was greenish-black, the acidity and sulfate concentration were lower than in the Upper Pond and sulfate-reducers were always recovered in higher numbers. Free H_2S was detected in the water only when the color was greenish-black. The color was undoubtedly due to precipitation of FeS which is in agreement with lower iron concentration found in the Lower Pond.

It is interesting to note that dissimilatory sulfate-reducers are active in a highly acidic environment. The two cultures which have been isolated do not reduce sulfate in artificial media in the laboratory when the pH is below 5.5. However, sulfate is reduced in the laboratory using sawdust as a substrate in a mixed culture system at a pH of 2.8. It is possible that a microenvironment of higher pH is set up around wood or other suspended particulates in a system having a low pH.

It must be emphasized that sulfate reduction cannot occur in the absence of organic materials since sulfate-reducing bacteria are clearly heterotrophic. The substrate range for these bacteria consists chiefly of fermentation products,

and not sugars or polysaccharides. Recalling that the Lower Pond contains soluble sugar and that sugars believed to be glucose and xylose disappeared in the depths of the sawdust, it seems clear that a cellulose degradation process involving several physiological types of microorganisms is required to provide substrate for the sulfate-reducing bacteria. The data presented in Table 6 gives some insight into this process.

In the 22 C or 37 C cultures containing fresh wood dust and either distilled water or acid mine water, reducing power (Nelson's) increased more than anthror measurable sugar. This suggests that sugars in relatively long chain lengths are degraded to smaller fragments more rapidly than insoluble wood dust is converted to soluble sugars. This is especially true of the 37 C culture which contained acid mine water At 14 days, the ratio of reducing sugar to anthrone sugar is greater than 1. It also suggests activity of mesophilic fermenting microorganisms at 37 C in fresh wood dust, and indicates that the organisms responsible are strict anaerobes. It must be emphasized that acid had no effect upon cellulose degradation in this system, and that H_2S present had no effect upon the determination of reducing sugars. Then, the difference in reducing power between the distilled water and acid mine water, fresh wood dust cultures can be attributed to a lowering of the O/R potential by H_2S, and the effect of the lowered O/R potential on the bacteria responsible for the fermentation process. The ratio of reducing sugars to anthrone sugars (greater than 1:1) may also indicate the presence of aliphatic aldehydes and ketones in units of 4 carbons or less as opposed to compounds of 5 carbons

or more, which are required to react with anthrone reagent.

In contrast to 37 C fresh wood dust cultures, the supernatants of 50 C fresh wood dust cultures show a greater buildup of anthrone sugars than of reducing sugars. This suggests the activity of thermophilic cellulose splitting microorganisms upon the insoluble wood dust cellulose. The greater buildup of anthrone sugars in the acid mine water culture, in which sulfate reduction was occurring, also indicates that this may be an anaerobic process.

The lack of change in anthrone or reducing sugars at 22 C and 37 C cultures, which contained partially degraded wood dust and either distilled water or acid mine water, suggests a steady degradation sequence: insoluble wood dust → anthrone sugar → reducing sugar → fermentation products which were utilized by sulfate-reducing bacteria.

At 50 C, in both cultures which contained partially degraded wood dust, reducing sugars increased over anthrone sugars. These data suggest that the primary activity of thermophilic cellulose splitting microbes in partially degraded wood dust was the cleavage of soluble degradation products to smaller units. The buildup of reducing materials at 50 C but not at 37 C or 22 C suggests that mesophilic bacteria were responsible for their further degradation.

It is clear that the microorganisms isolated from the wood dust do not represent the overall activity. Any or all of these species, particularly the Streptomyces species which degraded cellulose, may be important to the sulfate reducers, not only as a source of nutrients but also as metabolic agents for lowering the O/R potential. This is also true of the heterotrophic

Lower Pond isolates.

It is quite surprising to find higher numbers aerobic iron and sulfur oxidizing bacteria present in the Lower Pond under generally anaerobic conditions when compared to the Upper Pond. We must assume they are increasing in numbers in the Lower Pond. This may reflect the availability of ferrous and sulfide ions as energy sources. We have not examined the isolates for the possibility of facultative autotrophic growth on sulfur and organic compounds. This is not likely because the iron oxidizers outnumber the sulfur oxidizers by 10x and the iron oxidizers are considered to be obligate autotrophs. Origin of the oxygen requirement for autotrophic iron and sulfide oxidation is a puzzling question in this environment. We do not understand how O_2 exchange at the air water interface can satisfy the organisms O_2 demand in the presence of detectable H_2S. One possibility is a cyclic presence and absence of O_2 which could be related to photosynthetic cycling. Numerous Euglena cells are observed microscopically in wet mounts prepared from Lower Pond water These organisms may liberate O_2 during daylight and the system would be anaerobic during dark periods allowing sulfate reduction to proceed. Our data however does not suggest such fluctuations since H_2S is detected in the light. Another possibility is the simultaneous growth resulting from a symbiotic association of sulfate reducers, iron and sulfur oxidizers and photosynthetic Euglena.

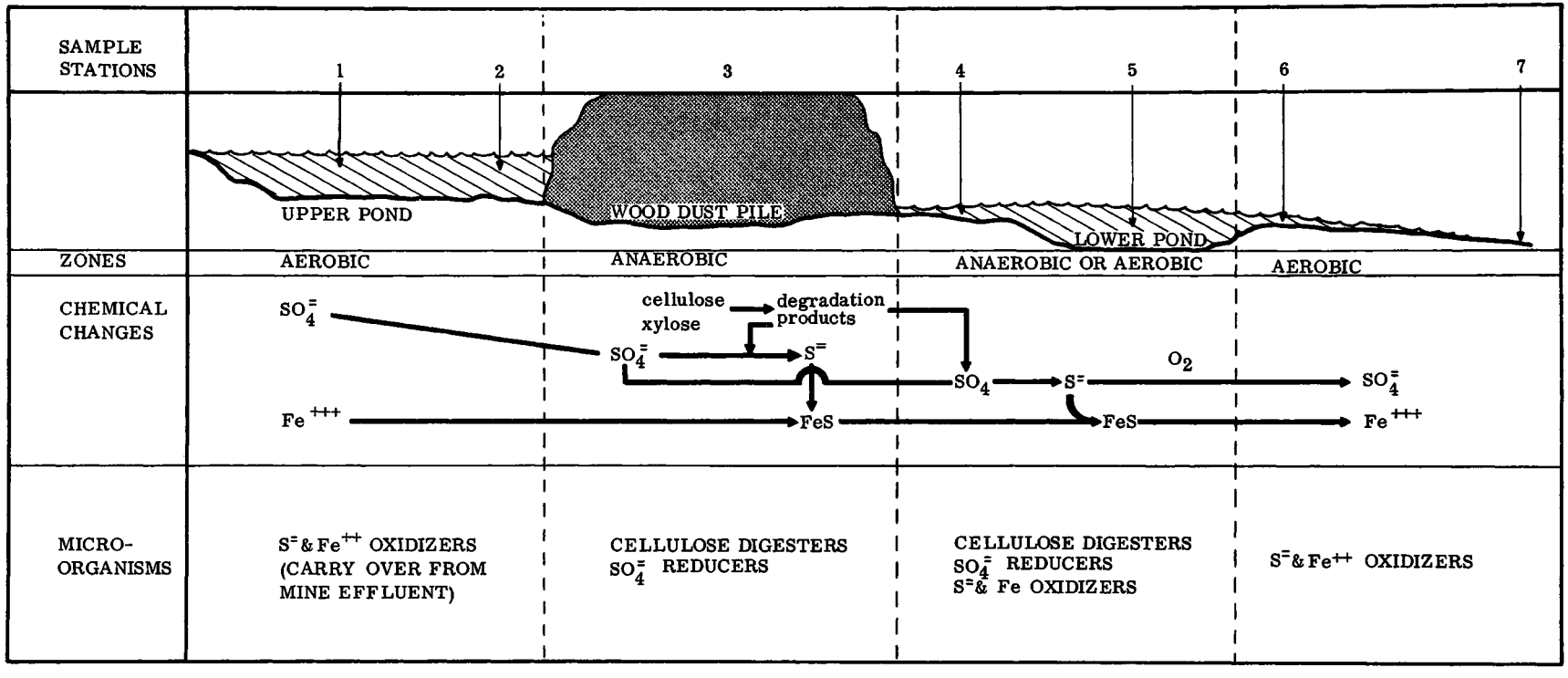

Fig 38

Schematic outline of the acid stream system depicting the distribution of chemical changes and microorganisms along the 7 sample locations.

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VI. MICROBIAL SULFATE REDUCTION IN ACIDIC MINE WATER AND ITS POTENTIAL UTILITY AS A WATER POLLUTION[1]

The activities of microorganisms in streams contaminated by acidic mine effluents have previously been described (5,6). Two of the organisms observed to have activity were anaerobic bacteria which reduced sulfate. The energy supply for sulfate reduction was derived from wood dust under natural conditions and the sulfate was supplied as the result of the metabolic activities of autotrophic Thiobacillus species acting on pyritic materials. Under controlled laboratory conditions, e.g. temperature and wood dust concentration, sulfate could be removed from acid mine effluents with a concomitant increase in pH(6).

In an earlier investigation, Moulton et. al. showed that mixed cultures of sulfate-reducing bacteria increased the pH of a lactic acid - mineral salts medium containing sulfuric acid from 5 to 8.9 in 8 days at room temperature (2). It was proposed from these data that wood dust or sewage might provide nutrients and a sufficiently low O-R potential for the natural establishment of sulfate-reducing bacteria, and that these bacteria could be used to dispose of sulfuritic waste materials in water of the type that discharges from abandoned coal mines.

This report considers sulfate reduction in mixed culture systems in which sulfate is supplied by acid mine drainage or $MgSO_4$ salt solutions and carbon is supplied as wood dust. Particular emphasis is placed on the physical and chemical parameters which appear to be pertinent to the activity of

[1] Submitted for publication to Applied Microbiology.

sulfate-reducing bacteria in acid mine water.

MATERIALS AND METHODS

Chemical Determination Total dissolved iron and sulfate were determined as previously described (5). O-R potentials and pH were measured with a Corning expanded scale pH meter. (Corning Glass Co., Corning, N. Y.)

Media and Growth Conditions Sulfate-reducing bacteria were enumerated by a modification (5, 6) of Postgate's method (3).

Enrichment cultures of sulfate-reducing bacteria which contanined mixed populations were partially purified by isolation on plates of Desulfovibrio desulfuricans medium no. 3 (3) which had been overlayed with 1.5% water agar. Black colonies which appeared after incubation at 37 C in 1 atm of 95% N_2-5% CO_2 were inoculated into 2 oz. prescription bottles containing medium "C" of Butlin and Adams (1). The cultures were incubated at 37C and then held at ambient temperature for future use.

Wood Dust Cultures Wood dust-acid mine water cultures were prepared by placing 1 liter of water (pH 3.6) obtained from an abandoned drift mine (Ohio #47) and 400g of wood dust into a 2-liter Erlenmeyer flask. Wood dust was obtained from the surface (non-degraded) of a large wood dust pile and also from a depth of 3 ft. (partially degraded wood dust) into the pile. The pile was adjacent to a mill which cut hardwoods - primarily oak. Cultures containing each wood dust sample

were incubated at 25 ± 2C, 37C, and 50C. A duplicate set of cultures in which 890 µg sulfate (as $MgSO_4 \cdot 7\ H_2O$) per ml of water replaced acid mine water was incubated as above. The wood dust tended to settle during incubation and the overlying solutions were periodically examined for sulfate, iron, and hydrogen ion concentrations.

Interrelationships Among Chemical Parameters Flask cultures containing partially degraded wood dust and acid mine water were prepared as described above. Sodium lactate was added to the flask to give a 0.1% concentration (w/v) and the mixed cultures were seeded with 25 ml of a 7-day culture of mixed sulfate-reducing bacteria to increase the population of sulfate-reducing bacteria. The cultures were incubated at 37C. O/R, pH, dissolved iron and sulfate concentrations were determined at suitable time intervals in a 25 ml aliquot which was removed aeseptically. Care was taken not to agitate the aliquot prior to O/R determination.

Addition of Exogenous Substrates Flask cultures containing 400 g of partially degraded wood dust and 1 liter of $MgSO_4$ solution (800 µg sulfate per ml of water) were prepared as described above. Individual culture flasks were supplemented with various carbon sources as follows: 0.01%, 0.1% and 1.0% glucose; 1.0% xylose; 0.1% and 1.0% sodium butyrate; 0.1% propionic acid; 0.1% acetic acid; 0.1% sodium formate, 1.0% succinic acid; and 0.1% acetone. One flask which did not contain an added carbon source was held as a control. The cultures

were incubated at 37C and aliquots were assayed for sulfate at suitable time intervals.

RESULTS

The effect of temperature and wood dust condition on sulfate reduction.

Table 13shows the effect of wood dust condition (fresh vs partially degraded from 3 feet in the wood dust pile) in sulfate removal from acidic mine water. Sulfate removal implies sulfate reduction to sulfide and either precipitation of sulfide as black FeS or loss from the system as H_2S gas. Table 13also shows the effect of incubation temperature on sulfate concentration in wood dust cultures containing acid mine water (initial pH 3.6 to 3.8). More sulfate was reduced at 37C than either ambient temperature or 50C over a 14 day period. Cultures at 50C reduce sulfate more rapidly and to a greater total extent than do ambient cultures, regardless of the wood dust condition. Although maximum total sulfate reduction occurred at 37C in the presence of partially degraded wood dust, the maximum rate of sulfate reduction occurred at 37C when fresh wood dust was present. This suggests that the fresh wood dust initially contained a greater amount of carbon available to the sulfate reducers and that this was rapidly spent without adequate replenishment from cellulose decomposers. The partially decomposed wood dust appeared to allow a more consistent rate of sulfate reduction over a longer time period.

The influence of substituting $MgSO_4$ (initial pH 4.2-5.8) in place of acid mine water is shown in Table 14 when values are compared to those in

Table 13. A comparison of the effect of wood dust quality (i.e. the extent of wood dust degradation by microorganisms) and incubation temperature on the reduction of sulfate and removal of H from acid mine water in untreated wood dust cultures containing 400 grams of wood dust and 2 liters of water. Each culture contained acid mine water with an initial sulfate concentration of 890 ug

	Non Degraded Wood Dust			Partially Degraded Wood Dust		
Incubation Temperature	Ambient Temp. 22 ± 2 C	37 C	50 C	Ambient Temp 22 ± 2 C	37 C	50 C
ug Sulfate removed per ml of water after 14 days	30	480	65	290	590	460
Maximum rate of sulfate removed in ug/ml of water per day	10.0	71.7	30.0	19.4	53.2	38.8
pH range 0 days to 14 days	3.6-4.2	3.7-5.8	3.8-4.2	3.5-4.3	3.5-6.4	3.7-5.8

Table 14. A comparison of the effect of wood dust quality and incubation temperature on the reduction of sulfate and removal of H^+ from $MgSO_4$ solution containing 400 grams of wood dust and 2 liters of water. The initial sulfate concentration was 890 ug per ml of water.

	Non Degraded Wood Dust			Partially Degraded Wood Dust		
Incubation Temperature	Ambient Temp. 22 ± 2 C	37C	50 C	Ambient Temp.	37 C	50 C
ug Sulfate removed per ml of water in 14 days	0	715	140	290	700	480
Maximum rate of sulfate removal in ug sulfate removed per ml of water per day	0	80.0	56.0	27.5	58.3	62.5
pH Range 0 days to 14 days	4.2-4.7	4.2-5.0	4.5-4.8	5.8-6.4	5.8-7.3	5.2-7.3

Table 13 When partially degraded wood dust was the substrate for the organisms there was a slight increase in sulfate reduction values in $MgSO_4$ at ambient temperature and at 50C and a somewhat greater increase at 37C. There was a marked increase in both the rate and total amount of sulfate reduced when $MgSO_4$ was substituted for mine water at 37C and at 50C when fresh wood dust was the carbon substrate. This may be attributable to the initial pH difference. No sulfate reduction was observed at ambient temperature in fresh wood dust. This may be due to lack of nutrients normally found in acid mine water which are required by the mesophilic wood dust decomposers. The iron concentration in the $MgSO_4$ cultures was never in excess of 2.5 µg/ml and was present as a constituent of the wood dust but not added intentionally.

Chemical and Physical parameters during growth in mixed culture.

Figure 39 shows changes in four parameters during growth of the mixed culture system in wood dust - acid mine water which had been enriched with 0.1% sodium lactate. The pH increased from 3.6 to 7.0 during a 10 day period. Eh (O/R) continually decreased but had a change in rate of decrease at about the 4th day. The solution potential became negative between the 4th and 5th day.

At approximately the same time the slope of the pH curve increased and an abrupt alteration in the rate of sulfate removal became evident. The concentration of dissolved iron increased rapidly for the first 6 days which can be attributed to increased solubility of ferrous iron as the potential dropped. After 6 days the Eh approached - 200 millivolts and the sulfate removal

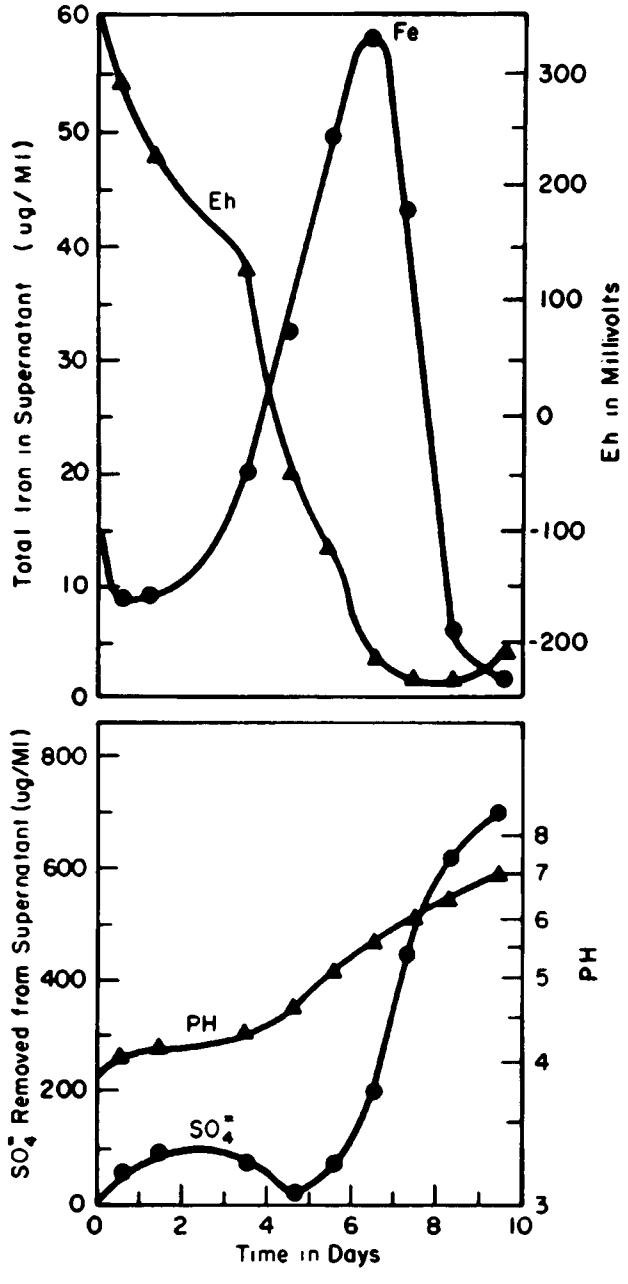

Fig 39

The relationships among pH, Eh, iron concentration (both ferrous and ferric), and the amount of sulfate reduced in the supernatant of a wood dust culture containing 400 grams of partially degraded wood dust and 2 liters of acid mine water (900 ug of sulfate per ml of water). Prior to incubation the culture was enriched with Na-lactate to a final concentration of 0.1% (w/v) and seeded with a 25ml aliquot of a mixed culture of sulfate-reducing bacteria.

proceeded at a maximum rate. Iron decrease was concomittant with sulfate reduction during this time period because of precipitation of black FeS.

Exogenous substrate suppliments

The effect of carbon substrates added to wood dust cultures in sulfate removal is presented in Table 15. Several substrates increased sulfate reduction while others retarded or inhibited reduction. Glucose in a concentration of 0.1% stimulated sulfate removal whereas a 1.0% concentration actually retarded sulfate removal when compared to a control. Incubation of the glucose culture for an additional 12 days does not permit sulfate reduction. Sodium formate allows sulfate reduction, but never at the rate of the control culture. Although 18 day incubation of the succinate culture is not sufficient to cause sulfate reduction, prolonged incubation results in a rate of sulfate reduction greater than that in the control culture.

MPN determinations of sulfate-reducing bacteria made after a 12 day incubation period correlate with sulfate reduction. These data are shown in Table 16.

The inhibition or retardation of sulfate reduction appeared to be due to either an increased lag period or a different rate of removal. Figures 40 and 41 present curves showing sulfate removal versus time in wood dust - acid mine water cultures which have been supplemented with various carbon sources. Figure 40 shows that lactic acid and succinic acid increases the rate (slope) of sulfate removal as compared to control curves. However, a long lag period is induced in the presence of succinic acid. This may be due to

Table 15. The effect of the addition of exogenous substrates on sulfate reduction in wood dust cultures. Each culture contained 400 grams of partially degraded wood dust and 2 liters of an $MgSO_4$ solution adjusted to 800 ug sulfate per ml of water. Substrate were added to give the final % concentrations (w/v) shown (and incubated) at 37 C. The % increase in sulfate reduced in the culture containing an exogenous substrate and a control culture which contained only 800 ug Mg SO_4 per ml of solution and wood dust. Values are expressed as % increase in sulfate reduction over the control. Inhibition indicates that less sulfate was reduced in the culture containing added substrate than in the control culture.

SUBSTRATE	Concentration as %	% increase of sulfate reduction in an 800 ug/ml $MgSO_4$ solution after 14 days incubation at 37° C
GLUCOSE	1.0	0 % (Inhibition)
GLUCOSE	0.1	83 %
GLUCOSE	0.01	0 %
XYLOSE	1.0	0 % (Inhibition)
LACTIC ACID	1.0	173 %
SODIUM LACTATE	0.1	70 %
SODIUM BUTYRATE	0.1	137 %
PROPIONIC ACID	0.1	34 %
ACETIC ACID	0.1	51 %
SODIUM FORMATE	0.1	0 % (Inhibition)
SUCCINIC ACID	1.0	0 % (Inhibition)
ACETONE	0.1	0 %

Table 16. Enumeration of sulfate reducing bacteria in cultures after 12 days at 37 C.

Substrate in addition to wood dust	MPN/100ml
1.0% Glucose	2.3×10
1.0% Succinic Acid	9.1×10
1.0% Lactic Acid	4.6×10
None	4.6×10

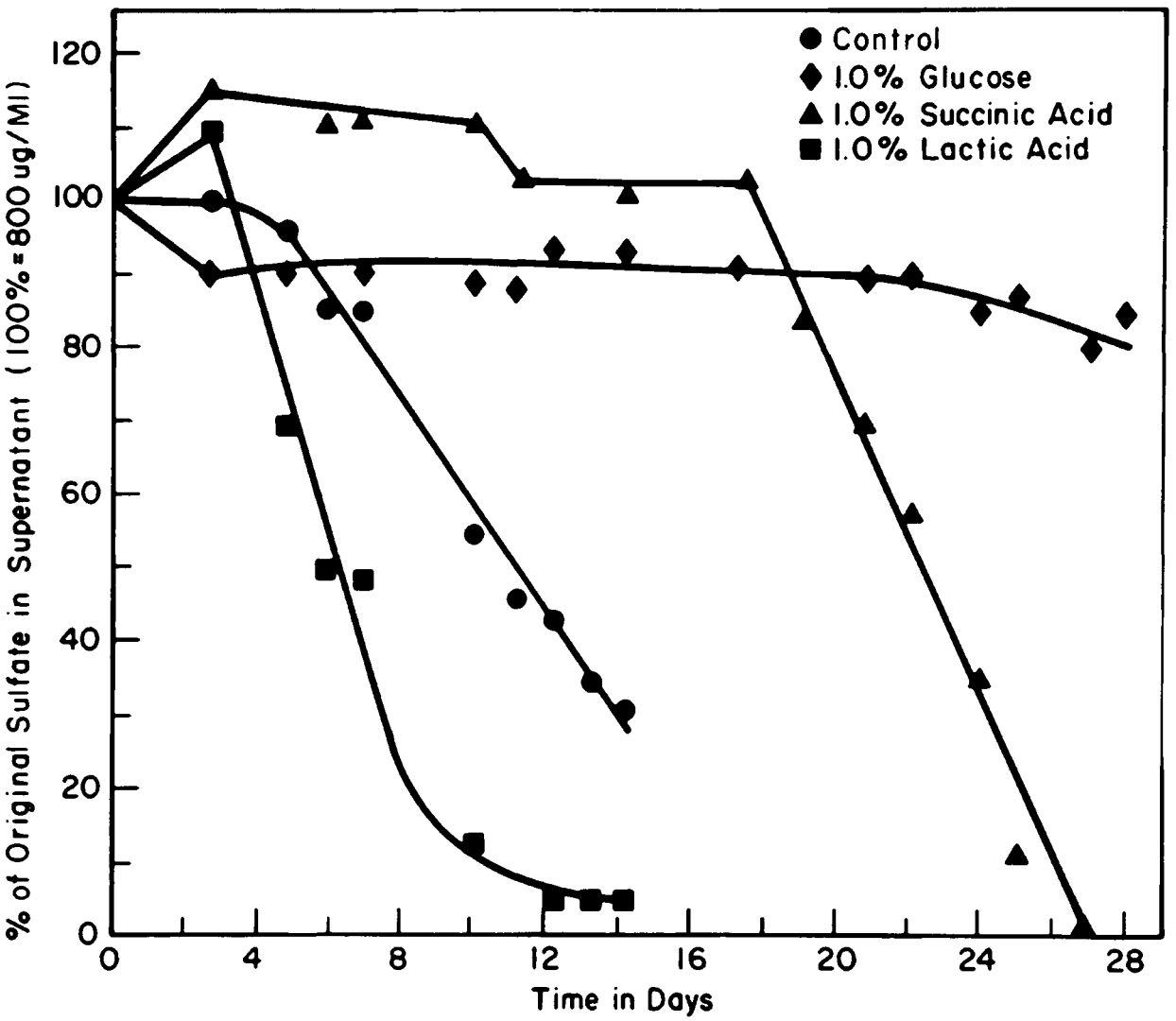

Fig 40

The influence of 1.0% lactic acid, 1.0% succinic acid, and 1.0% glucose on sulfate reduction in partially degraded wood dust-acid mine water cultures at 37°C.

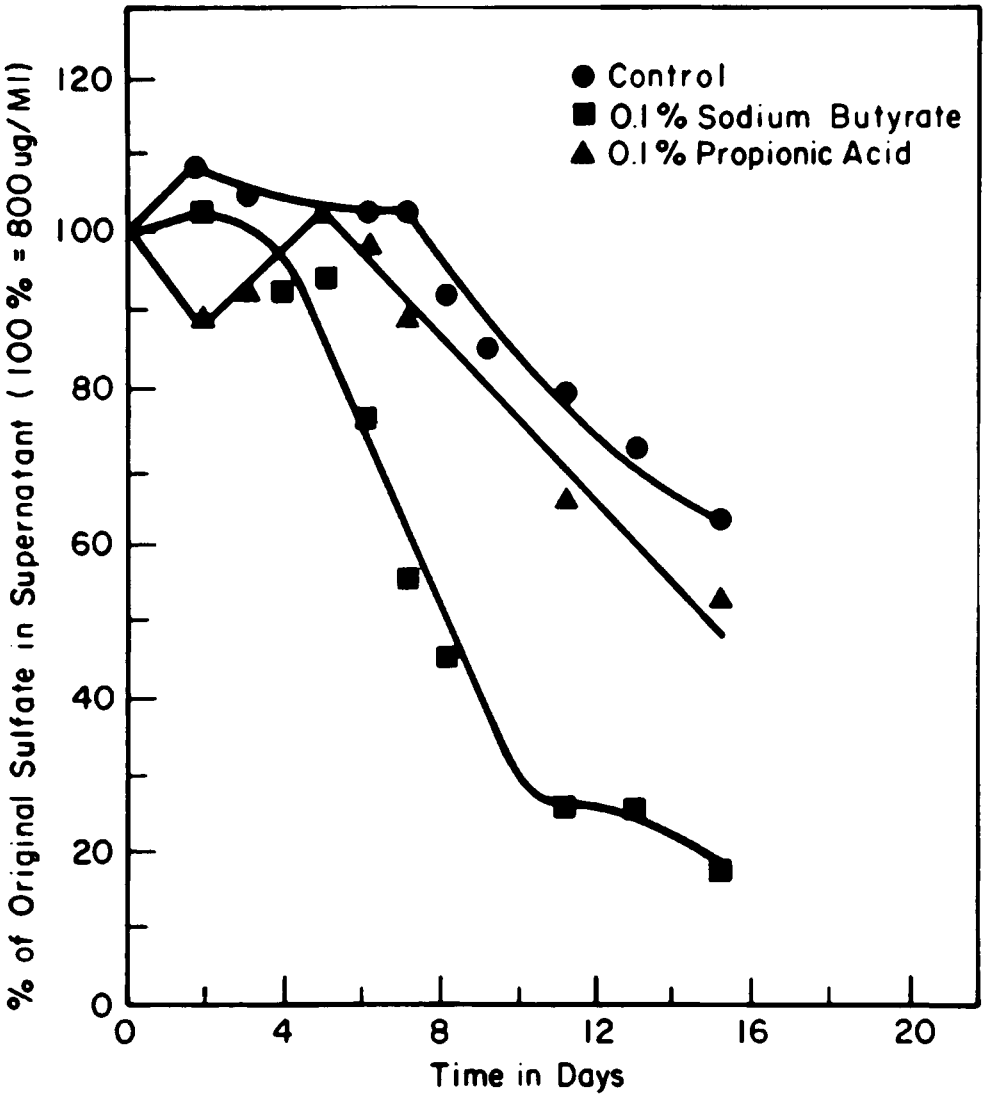

Fig 41

The influence of 0.1% sodium butyrate and 0.1% propionic acid concentration on sulfate reduction in partially degraded wood dust-acid mine water cultures at 37°C.

increase in O/R potential rather than a direct inhibitory effect and also indicates that data presented in Table 15 must be interpreted with caution. Figure 41 shows results of an experiment which was essentially the same as that shown in Fig.40 except that different carbon sources were added. The similar slopes observed with propionate and the control indicate that propionate slightly stimulated sulfate reduction, by decreasing the lag period. Butyrate stimulated sulfate reduction by both decreasing the lag period and increasing the rate of reduction (slope).

DISCUSSION

Two primary difficulties must be overcome to accomplish sulfate reduction in acidic waters. Dissimilatory sulfate-reducing bacteria require an O-R potentil of -150 to -200 MV (4), therefore the water must be made anaerobic. Secondly, a source of organic nutrients to supply energy and carbon for the heterotrophic anaerobis is required. Although we have previously reported (5) that numbers of anaerobic microorganisms in acid mine water are low, the addition of organic materials is favorable to the establishment of anaerobic heterotrophic microflora in acidic water (6). This process is illustrated graphically in Fig. 39. Wood dust degradation is necessary for the establishment of an anaerobic microflora and, in particular, sulfate-reducing bacteria. The rate of wood dust degradation therefore appears to control both the initiation of and the rate of sulfate reduction. Several physiological types of microorganisms (both aerobes and anaerobes) undoubtedly participate in the wood dust degradation process according to proposed degradative

schemes presented previously (6).

We have also reported (6) that temperature is a primary factor affecting wood dust breakdown and that the temperatures most favorable for this process may not yield maximum rates of sulfate reduction. This is a result of differing temperature optima among the responsible physiological groups of bacteria. Whereas the sulfate-reducing bacteria in our system grew best at 37C, wood dust degradation occurred most rapidly at 50C.

The energy requirements of the sulfate-reducing bacteria must also be considered. Both butyrate and lactate served as energy sources for our isolates in artificial culture media (unpublished data). Other sulfate-reducing bacteria have been shown to be capable of oxidizing straight chain alcohols, pyruvate, and choline (4) and acetate is commonly formed as the end product of the fermentation. The data (Table 15 therefore, suggest that acetate is recycled in the wood dust system. This may be accomplished by other anaerobes, most likely Clostridium species. The same explanation may fit the observed enhancement of sulfate reduction by propionic acid.

The system may be upset by the excess addition of substrates which enhance sulfate reduction in lower concentrations. This is shown by the concentration phenomenon observed for glucose (Table 16). This effect may be a result of an interruption in the cellulose breakdown process, resulting in a shift in microflora which do not produce substrate for sulfate-reducing bacteria. Note that viable sulfate-reducing bacteria are present in cultures in which measurable sulfate reduction did not occur (Table 16)and that these anaerobes do not attack glucose (4).

Wood dust quality also influences the rate of sulfate-reduction. The maximum rates of sulfate reduction occurred in non-degraded wood dust at 37C (Tables 13 and 14). Because a longer lag period was observed in fresh wood dust cultures than in partially degraded wood dust, the apparent equality of total sulfate removal in all 37C cultures is misleading. In practical use, the lag period is not an important consideration since the conditions under this circumstance are more similar to a continuous culture than a batch culture.

The data suggest (Table 14)that iron is not required in dissimilatory quantities for sulfate reduction in the mixed culture system. Sulfate-reducing bacteria do, however, require iron (4). Constant influx of acid mine water into the system would be expected to furnish sufficient iron and sulfate for these bacteria.

Acid mine water which has been treated by the wood dust process is not potable. Although much of the iron and sulfate have been removed, further water treatment is necessary. Precipitated iron sulfides in a mine water system must be removed in order to prevent natural reoxidation of iron and sulfide (as well as oxidation resulting from Thiobacillus species which are indigenous to acid mine drainage) if practical use is to be made of the process. One possible inducement may be with recovery of sulfur as FeS or as Sulfur if an additional microbiological conversion step was introduced into the system. Although no data is presented in this paper it has been possible to remove sulfate in a continuous system using 5 gallon holding tanks for the wood dust culture and flowing acid mine water through the culture.

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6. Chapter IVthis report.

VII. GENERAL SUMMARY AND CONCLUSIONS

Formation and Prevention of Acid Pollution Originating from Bacteria.

The role of iron and sulfur oxidizing bacteria in the production of iron, sulfate and hydrogen ions from pyritic minerals has been extensively considered elsewhere. This report verifies earlier conslusions and considers the autotrophic iron and sulfur oxidizing bacteria only from an ecological viewpoint. Distribution of such organisms in the environment has led us to conclude that biological production of acid occurrs in gob-piles and in mines but is not primarily produced in streams or other receiving waters. Consequently, acid builds up in the gob-pile (or mine) at a somewhat consistent rate over an extended time period. The acid appears to be flushed out of the site of origin via rain water, which causes a sudden surge of acidity in a receiving stream. If the rainfall was prolonged, a dilution effect would result in decreased acidity in the stream.

This type of pollution therefore originates at rather localized sites (as compared to streams) and appears to a large extent to be caused by activities of living bacteria. This further suggests that preventative methods which utilize antimicrobial agents should prove sucessful at specific locations. That is, success of prevention of this type pollution would depend upon ability to inhibit causative bacterial metabolism at the origin. Location and inhibition of microbial activity should not be problematical in the case of gob-piles but may quite difficult in the case of abandoned drift mines.

The type of antimicrobial agent to be employed in a field situation is a matter that must be further explored. Some fruitful investigations with regard to

inhibition of iron and sulfur oxidation by autotrophic bacteria have been carried out in this laboratory on programs not included in this report. However, preliminary evidence suggest that chemical inhibitors might be practical with reference to cost, availability and lack of toxicity for organisms other than the iron and sulfur oxidizers. Further work in this direction is warranted and needed.

Influence of Mine Drainage in Microflora of "Normal" Stream.

Mine drainage is toxic to the vast majority of microbes and higher life forms (e.g. insects, fish etc.) which normally inhabit non acid streams. However, a small acid tolerant population of heterotrophic microbes exists in streams, which survive and persist rather severe acid conditions (pH 2.5 - 2.8). Indeed, data presented in Chapter IV of this report, show that pH 2.8 acid drainage supports a much higher heterotrophic population (both in terms of numbers of individuals and numbers of species) than was heretofore suspected. The organisms appear to be indiginous to and proliferate in pH 2.8 water and they also proliferate under neutral conditions. The implication in this finding is that by-products of heterotrophic metabolism would, in general, tend to increase pH (lower acidity) and would ultimately allow a succession of higher life forms to become established. That is, streams would return to a normal condition.

The best estimate of acid abatement necessary to allow this natural process to proceed is a rise to pH 5. This conclusion will vary depending upon volume of drainage in proportion to volume of receiving water. It should be emphasized that hydrogen ion (acid) is the parameter of paramount importance with lesser

significance attached to sulfate ions, insofar as toxicity of mine drainage to heterotrophic microorganisms is considered. Iron ions in the range 1 to 100 ug/ml did not adversely influence microbial growth. Therefore, from the viewpoint of overall biological activity iron should not be a major consideration. Iron concentration would normally be less than 100 ug/ml when the acidity is above pH 4.0. This conclusion does not, however, consider problems other than biology (e.g. corrosion, aesthetics etc.). Because of the above considerations, abatement methods based upon removal of sulfate and hydrogen ions would be desireable, if methods were available.

Iron, sulfate and hydrogen ion Removal by Microorganisms and Its Potential as an Abatement Method.

Reduction of sulfate ion to sulfide is a well recognized activity of anaerobic bacteria in the genera Desulfouibrio and Desulfotomaculum. This process has been shown to occurr in acidic mine water, providing a utilizable carbonaceous energy supply is made available to the microorganisms. During the process of microbial sulfate reduction, iron is removed via precipitation and a net loss of acidity also results. The process can be manipulated in the laboratory to increase the overall efficiency and attempts to scale up the process seem to be sucessful. This suggests that such a process could be developed into a practical abatement method at specific locations. Potential methods for accomplishing this process are lagooning, design of a facility similar to those used for anaerobic sewage digestion, and conversion of certain mines into anaerobic mines where the reduction process would proceed directly in the mine. This last suggestion has considerable

potential because by products of metabolism of many anaerobic bacteria are toxic to the aerobic Thiobacillus - Ferrobacillus species which oxidize pyrite. The net effect would be to suppress formation of acid in the mine while removing acid and sulfate already formed.

The carbonaceous energy source for sulfate reducers was wood (saw) dust in the experiments described in Chapters IV and V. Activity of a third group of bacteria was essential to accomplish this process. The third group, referred to as cellulose digesters, also proliferates under acidic conditions and any low cost cellulose should be able to supply nutrients to the sulfate reducers. For example, straw, sewage, waste paper, etc., are all potential sources of carbon for sulfate removal using microbiological processes.

VIII RECOMMENDATIONS

A significant amount of acid mine drainage appears to be produced via microbial metabolism and should therefore be amenable to abatement by utilizing antimicrobial agents. We recommend that studies be undertaken to determine the feasibility of scaling up laboratory experiments to effectively inhibit or control acid production in the field where sites of Thiobacillus and Ferrobacillus activity can be identified (e.g. gob piles). These studies should be on a pilot scale and should consider effects of the antimicrobial agents on other components of the ecosystem, persistence in the environment, effectiveness, cost, etc. Laboratory investigation into the mode of action of agents inhibitory to the Thiobacillus - Ferrobacillus group of bacteria should be continued because it serves to determine the mostsuitable and effective substances to be used in the field.

The ability of sulfate reducing bacteria to reduce sulfate to sulfide under acidic conditions should be exploited in specific situations particularly since acid is removed and sulfate can be an adverse influence to some biological systems in conjunction with acid. Practical methods of harnessing this biological process should be investigated and should include the following possibilities: (A) design of an anaerobic treatment facility similar in principle to those used for domestic waste treatment (B) design of lagoons (C) actual use of mines as fermentation vessels.

Investigations designed to increase the efficiency of the biological conversion

of sulfide to sulfate should be supported. This type investigation should consider potential carbon or energy sources for the bacteria. It may be possible to benificially combine problems associated with solid waste removal to acid mine drainage abatement, through use of waste paper and other fibrous carbon materials. That is, we see no reason why sewage, algae or any waste vegetable matter could not be substituted for sawdust in the process described in this report. Potential recovery of by products such as FeS or S from such systems should also be considered.

Since it does not appear likely that any single means of prevention or abatement will be practical or that the acid drainage problem will be completely controlled in the near future; we recommend that studies be continued in an effort to understand how much acid organisms can tolerate and how heterotrophic organisms grow under extreme acid conditions. Such studies will be of benefit in determing the extent of abatement that is necessary in a specific situation.

IX Publications Resulting from this Research Project.

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Jon H. Tuttle. 1966. "A study of the Activities of micro-organisms in acid mine water." 189 pp.

Bruce McCoy. 1967. An investigation of the relative influence of iron, sulfate and hydrogen ions on the microflora of a non-acid stream.

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